Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 84 NOVEMBER, 1953 No. 2

SECTION MEETINGS

BALTIMORE	
Camp Detrick, Md.	October 23, 1953
ILLINOIS	
Searle & Co.	October 20, 1953
MINNESOTA	
Mayo Foundation	October 22, 1953
SOUTHERN CALIFORNIA	
University of Southern California	October 15, 1953

Effect of Cortisone on Tissue Mast Cells in the Hamster Cheek Pouch.* (20609)

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Tissue mast cells are now of interest because of the belief that they may be unicellular endocrines which release heparin(1,2), histamine(3), and hyaluronic acid(4). The dramatic results of the use of cortisone in the treatment of rheumatoid arthritis(5) have stimulated inquiry into its action on various tissue elements including mast cells. Investigators have stated that cortisone acts as an inhibitory factor on these cells as on other cellular components of mesenchymal tissues (6). Mast cell tumors in dogs have been successfully treated with large doses of cortisone(7), and the neoplastic mast cells showed cytoplasmic vacuolization, conglomeration, altered staining reaction of metachromatic granules, disappearance of granules, fraying and disruption of the cellular membrane, granule scattering and finally cellular destruction and disappearance. However, Kelsall and Crabb reported that cortisone in the hamster did not affect the number of mast cells in the sublingual glands(8) or in the thymi(9).

The present report is concerned with the effects of cortisone upon the mast cells of the cheek pouch of the hamster.

Method. Cortisone acetate (Merck) was injected subcutaneously into 12 adult hamsters (*Mesocricetus auratus*) in doses of 5 mg per day for periods varying from 12 to 23 days. The cheek pouches were excised under nembutal anesthesia (6-10 mg/100 g), and whole mount preparations stained with methylene blue were made by the method of Zahl and Nowak(10). The preparations were examined under the microscope at magnifications of 150x and 645x. Mast cell counts were taken at 150x in 5 different unit areas (0.3 sq mm) of each preparation, with the aid of a Whipple disc. As a control, the

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* Supported in part by a research grant to Boston University from the National Heart Institute of the National Institutes of Health, Public Health Service.

TABLE I. Mast Cell Counts in the Hamster Cheek Pouch and Clotting Times during Cortisone Treatment.

	Days treated	Mast cell count/5 unit areas (1.5 mm²)	No. of mast cells/mm²	Clotting time, sec.
Normals (avg of 4)		807	538	107
Cortisone-treated	12	302	201	
	14	139	93	
	14	567	378	
	14	404	269	
	14	272	181	60
	14	312	208	45
	14	197	131	60
	14	490	327	45
	14	443	295	60
	21	165	110	120
	21	110	73	30 min.
	23	116	77	
Avg of 12	12-23	293	195	

numbers of mast cells were counted in the cheek pouches of 4 untreated hamsters. Clotting time determinations were made on 7 hamsters by means of the Lee-White technique.

Results. A definite decrease occurred in tissue mast cells in the cortisone-treated hamsters as compared with the controls (Table I). The count in 12 hamsters during treatment averaged 293 per 5 discrete unit areas totalling 1.5 sq mm of the cheek pouch (195/sq mm), as compared with 807 (538/sq mm) in 4 normal hamsters. In the controls the mast cells were numerous, large, and both perivascular and extravascular in distribution. The presence of extruded granules indicated possible secretion. Cytological observations indicated the probable formation of mast cells from fibroblasts. In the treated hamsters, mast cells were fewer (especially remote from blood vessels), smaller, often spindle-shaped, and frequently adjacent to extracellular granules and amorphous stainable material. Somewhat similar changes have been described in the cheek pouch following x-irradiation (11,12).

The clotting times after 14 days of treatment with cortisone were less than the control value (Table I), consistent with the hypercoagulability reported from many clinical studies with cortisone. After 21 days of treatment, the clotting time increased, especially in one hamster (over 30 minutes). This might indicate a delay between the time of disruption of mast cells and the appearance of a significant increase in circulating anticoagulants.

Summary. Cortisone acetate injected subcutaneously into hamsters produced a decrease in the number of tissue mast cells in the cheek pouch. The size decreased and the cells were disrupted, with discharge of granules and stainable material. The treated hamsters consistently had fewer extravascular cells. The clotting times in the treated hamsters were at first somewhat less than in the controls, but in those surviving 21 days of treatment, an increase in clotting time occurred.

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Received October 7, 1953. P.S.E.B.M., 1953, v84.

Immunochemical Behavior of Tomato Bushy Stunt Virus.* (20610)

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Relatively little data exists on the immunochemical behavior of the plant viruses except for tobacco mosaic virus(1). Although unrelated to the latter in biological as well as in gross physical characteristics, tomato bushy stunt virus is comparable with respect to its high molecular weight (8 x 10^6). The availability of this spherical virus (26 mμ in diameter) in a purified crystalline state prompted the following study.

Methods and results. Bushy stunt virus was isolated from diseased tomato plants and crystallized according to the technic described by Stanley(2). Antiserum was prepared by immunizing rabbits with the virus according to the usual procedures. The quantitative precipitin titration technic of Heidelberger and Kendall, as carried out for study of tobacco mosaic virus(1), was followed. The results, shown in Table I, are similar to those observed for tobacco mosaic virus as well as for proteins of lower molecular weight. As antigen nitrogen is added in increasing amounts to a constant volume of antiserum, increasing amounts of total nitrogen are precipitated. Maximal total nitrogen precipitation, although not reached in this study, would undoubtedly occur in the region of antigen excess. Maximal precipitation of antibody nitrogen was found to occur within the equivalence zone. The antibody N/antigen N ratio is seen to be curvilinear with respect to added antigen N as also has been observed for tobacco mosaic virus. This relationship is apparently a function of high molecular weight.

Summary. Crystalline bushy stunt virus behaves as a typical protein in its immunochemical reactions.

TABLE I. Reaction of Bushy Stunt Virus with Homologous Rabbit Antiserum.

mg virus–N added/ml antiserum	Total mg N pptd/ml antiserum	Antibody N pptd/ml (by difference)	R*	Supernates tested for	
				Antibody	Antigen
.019	.053	.032	1.68	++	—
.038	.103	.065	1.71	++	—
.057	.152	.095	1.67	++	—
.077	.205	.128	1.66	++	—
.097	.260	.163	1.68	++	—
.100	.238	.138	1.38	++	—
.142	.351	.209	1.47	++	—
.190	.446	.256	1.35	+	—
.379	.854	.475	1.25	+	—
.386	.824	.438	1.13	+	—
.758	1.540	.782	1.03	±	—
1.138	2.128	.990	.87	—	—
1.517	2.568	1.051	.69	—	—
1.896	2.952	1.056	.57	—	—
2.275	3.256	.981	.43	—	—
2.548	3.384	.836	.33	—	—
2.749	3.696	.947	.34	—	—
3.839	4.440	.601	.16	—	±
4.169	4.624	.455	.11	—	±

* R = antibody N/antigen N.

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* This work was carried out during the tenure as Senior Fellow of the American Cancer Society, recommended by the Committee on Growth of the National Research Council, at The Rockefeller Institute for Medical Research, Princeton, N. J., 1946-1948.

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Received August 3, 1953. P.S.E.B.M., 1953, v84.

Effect of Androstene Derivatives on Metacorticoid Hypertension. (20611)

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Metacorticoid, or post-DCA, hypertension is a self-sustaining cardiovascular disease attained in rats approximately 2 months after the implantation of a desoxycorticosterone acetate (DCA) pellet and the substitution of saline for drinking water(1-4). In many respects this type of hypertension appears similar to essential hypertension in man(1,5), and responds to treatment with various drugs active in the human disease(6). Metacorticoid hypertension is abolished by hypophysectomy(2) or nephrectomy(3), but not by removal of the adrenals, thyroid, parathyroids, gonads, nor by drastic dietary restriction of Na or K(1).

Materials and methods. Each experimental group consisted of 4 male Sprague-Dawley rats which had received a single 20 mg DCA pellet 3 to 6 months previously, and had been maintained on 0.86% NaCl solution and Rockland Rat Diet *ad libitum*. The systolic blood pressures of treated rats were estimated with a photoelectric tensometer(7) immediately before and 2, 4, and 5½ hours after treatment, and were compared to simultaneous readings from solvent-injected controls. The mean blood pressure of over 400 normal rats by the tensometer method has been determined as 120 mm Hg, with a standard deviation of 10. Pressure readings on individual rats were made in ignorance of the treatment the rat had received and of the previous pressure reading. Each test was completed by one person on the same tensometer in a darkened room at constant temperature. The rats were trained for at least 3 months before testing and were deprived of food and saline during the course of the experiment. The significance of the results was determined by use of the one-tailed *t*-test(8). The following compounds were injected subcutaneously in corn oil or propylene glycol in doses of 20 mg/kg: testosterone, 4-androstene-3,17-dione, 11β-hydroxytestosterone, 11β-hydroxy-4-androstene-3,17-dione, 11-ketotestosterone, and 4-androstene-3,11,17-trione (adrenosterone). These compounds represented the following derivatives of 4-androsten-3-one: 11—H and 17—OH, 11—H and 17=O, 11—OH and 17—OH, 11—OH and 17=O, 11=O and 17—OH, 11=O and 17=O, respectively.

Results. The mean blood pressures for each group of rats are listed in Table I. The most active steroid was 11-hydroxyandrostenedione; it lowered the mean blood pressure 33-49 mm Hg over a 5½-hour period. Testosterone and its 11-keto derivative lowered blood pressure 18-22 mm over the same interval. Androstenedione and adrenosterone had depressor effects of approximately 9-18 mm, while 11-hydroxytestosterone was inactive.

Discussion. By comparing the structures of these compounds with their activities, it can be concluded that replacement of the 11-hydroxyl by a hydrogen or a keto group appeared to decrease the depressor activity of the 17-ketosteroids and increase the activity of the 17-hydroxysteroids.

In rats receiving DCA, testosterone temporarily reverses the increase of blood pressure(9), partially inhibits the increase in appetite for NaCl(10), and prevents nephrosclerosis and proteinuria(11), but not cardiorenal hypertrophy(12). The "protective" effect of testosterone has been ascribed to its renotrophic activity(13).

Adrenosterone, androstenedione, 11-hydroxyandrostenedione, and 11-hydroxytestosterone have less than 5% the anabolic and androgenic effect of testosterone propionate, as measured by the increase in the weight of the levator ani muscle and seminal vesicles of castrated rats(9). Apparently the action of these steroids against metacorticoid hypertension is not explicable on the basis of their anabolic or androgenic activities.

Summary. Six derivatives of 4-androsten-3-one were tested for depressor activity in

TABLE I. Effect of Androstenes on Hypertension.

Test	Derivatives of 4-androsten-3-one	Mean group blood pressure, mm Hg			
		Before inj.	2 hr*	4 hr*	5½ hr*
A	11β-hydroxy-4-androstene-3,17-dione	187	153†	140‡	138†
	Propylene glycol	187	186	177	187
B	4-androstene-3,11,17-trione	186	180	169†	178†
	4-androstene-3,17-dione	184	170‡	177‡	178‡
	Corn oil	182	188	186	190
C	11β-hydroxytestosterone	180	172	182	172
	Propylene glycol	181	180	182	178
D	11-ketotestosterone	183	168	160†	160†
	Testosterone	183	161‡	162†	164†
	Corn oil	178	180	182	182

* Hr after inj.
† P<.01 that treat mean = control mean.
‡ P<.05 ” ” ” = ” ” .

metacorticoid hypertensive rats. Replacement of the 11-hydroxyl by hydrogen or a keto group appeared to decrease the activity of the 17-ketosteroids and increase the activity of the 17-hydroxysteroids. The depressor activity could not be related to anabolic or androgenic activity.

The technical assistance of Catherine Tsou and Ardys Magner is gratefully acknowledged.

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Received September 22, 1953. P.S.E.B.M., 1953, v84.

Resistance Strain Gauge Arches for Direct Measurement of Heart Contractile Force in Animals.* (20612)

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While industrial uses of resistance wire strain gauges are highly diversified, the biologic application of this device has been limited almost entirely to the recording of hydraulic pressures. Actually there are numerous biologic changes of force which can be measured advantageously by this procedure which reacts isometrically and which, with the usual instruments, is capable of frequency recordings of 100 cycles/sec. In practice, it is only necessary to cement one of the standard coils to a metal strip which has elasticity characteristics suited to the force changes of the particular experiment. Garb(1), for instance, has used the principle to measure contractile force of an isolated papillary muscle. Robb(2) connected perfused, beating hearts of small mammals to the post of a Statham transducer and made similar records.

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* This study was supported by grants from the National Heart Institute, U. S. Public Health Service.

The present report describes the technic of preparing strain gauge arches which can be attached to the ventricles of large animals and made to record the force of each systolic contraction acting against the 2 points of attachment. Because of the unique compactness of this device, the chest wall can be closed after surgical attachment of the arch and lead wires brought through the wall for subsequent recordings in the post-operative animal. Recent experiments have established that the measurements of this method are representative of changes in different areas of both ventricles, are not directly influenced by variations in heart rate and are only moderately influenced by variations in arterial and venous pressure(3).

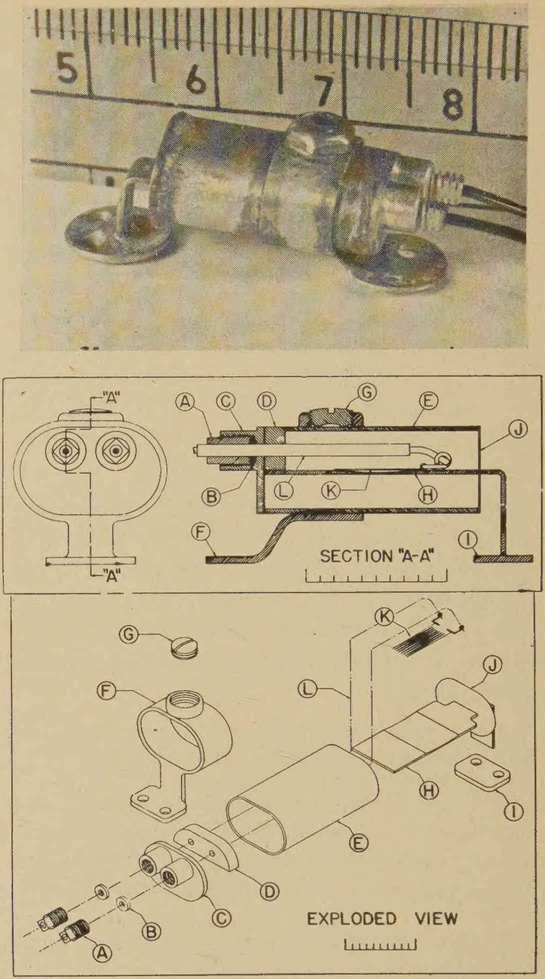

FIG. 1. Strain gauge arch with coil enclosed in brass tubing encasement. Above—photograph about twice actual size. Below—diagrammatic, sectional and exploded views with one cm scale. Parts designations as follows: (A) Stuffing box gland. (B) Stuffing box packing. (C) Body end integral with stuffing boxes. (D) Stop preventing upward movement of (H). (E) Tubular body or encasement. (F) Movable foot. (G) Set screw. (H) Elastic metal strip integral with rigid leg. (I) Foot. (J) Flexible metal diaphragm. (K) Strain gauge coil. (L) Lead wires.

Methods. SR-4 strain gauges type A-7 were used as pick-up units(4) and their output was carried to a strain analyzer and amplifier (BL-310) and direct inking oscillograph (BL-202) of the Brush Electronics Co. Two types of arches have been used. In one, the strain gauge coil is covered with insulating resins and, in the other, the coil is protected by a thin metal tube (Fig. 1). Although a variety of insulating resins have been tested, none were found which did not lose resistance rapidly in the animal's chest. This resistance breakdown occurred so much more rapidly in body fluids than in saline that saline immersion was not considered a useful guide. Ordinarily, with the resin protected coils, resistance between the coil and some free point on the arch would be greater than 1000 megohms. On the first post-operative day resistance usually dropped to fractions of a megohm, this being determined by connecting the meter to one lead of the coil and the other to the animal's mouth. In all cases, with resistance reduced to fractions of a megohm, the animal was grounded. This permitted satisfactory measurements for a few days and in some cases for a few weeks. With the metal protected coils, initial resistance would range in the area of 1000 megohms and would stabilize in the area of 25 to 100 megohms when tested during the period of use in the animal. Before using the strain gauge arches, they were customarily tested for sensitivity and their threshold of response determined in grams. Desirably, the threshold of response should be less than 10 g.

It can be noted in Fig. 1 that the arch with metal protected coil is additionally equipped with provision for adjusting the distance between the 2 "feet" or points which are to be attached to the ventricle. This permits, even in hearts of widely varying size, a standardized degree of diastolic tension in the muscle segment between the 2 points of attachment. This is accomplished by calibrating the arch, leaving unaltered all adjustments of the analyser and oscillograph and making recordings immediately after the arch is surgically attached but before the chest is closed. The degree of diastolic tension can then be recognized and expressed in grams. It has been shown previously that the force of systolic excursion increases about 5% for each 1% increase in initial diastolic length (stretch) (5).

The systolic force commonly recorded at the time of surgical attachment of the arches was usually in the range of 50 g. This necessarily decreases in chronic experiments due to gradual loosening of the attachments with resulting decrease in initial diastolic tension. To adjust for this, the sensitivity of the oscillograph recordings must be increased. In all cases with these closed-chest observations, results are expressed in terms of percentage change rather than in grams force. It has been shown in experiments with stimulant drugs, that the percentage change is not greatly influenced by the initial diastolic tension in control periods, or, correspondingly, by the measured systolic force in control periods, so long as these features remained within the common ranges(3).

Preparation of the arch with metal-protected coil. The arch with electrical components enclosed in a hermetically sealed metal tube was constructed as shown in Fig. 1. The tubular encasement or body (E) was made of yellow brass, the flexible diaphragm end (J) was made of thin silicon-bronze and the elastic member (H) with integral rigid leg was made of heat-treated beryllium copper. The brass button or foot (I) was soldered to this leg. The lead wires, specially selected to withstand frequent bending, were made of 14 strands of 1-mil copper wire covered with a vinyl chloride sheath and commercially designated as Tensolite W 1748(6). A particular feature of this completely insulated device is its lack of interference with simultaneous ECG recordings.

Operative technic. The operations were performed under pentobarbital anesthesia with positive pressure mechanical respiration and with liberal administration of antibiotics. The chest was entered anteriorly through the

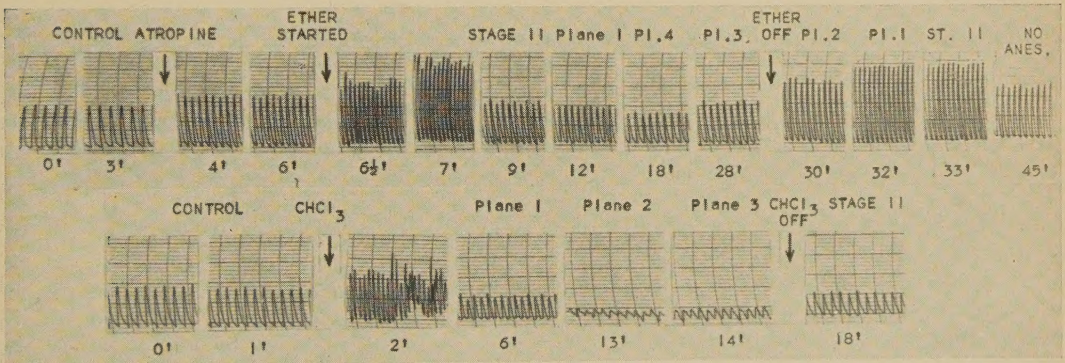

FIG. 2. Oscillograph recording of heart contractile force in chronically operated dog. Excursion amplitude is directly proportional to force. Anesthesia level (above) defined according to Guedel. Time (below) indicated in minutes. Ether anesthesia and chloroform anesthesia administered on successive days. Both experiments show increased and irregular force and rate during early struggling stage. Chloroform depression of contractile force is conspicuously greater than that with ether. During recovery from ether (in lighter planes of Stage III and in Stage II) there was a pronounced increase in contractile force considerably above control levels. This effect was frequently seen during recovery from anesthesia with all agents except chloroform and was tentatively ascribed to a sympatho-adrenal discharge.

fourth left intercostal space, the heart rotated to fully expose the anterior aspect of the right ventricle and the pericardium resected over this area. The arch was attached to the anterior surface of the right ventricle by cotton thread sutures, one set near the A-V sulcus and the other near the septum. Two or 3 sutures were laid at each point, threaded through the drilled holes in the feet of the arch and tied. Lead wires were brought out through the operative wound, the chest closed and aspirated. Chief complications in order of occurrence were adhesions, tearing out of sutures and breaking of lead wires. These complications were ordinarily diagnosed readily from the character of the tracing. The diagnosis was facilitated by the administration of epinephrine "test doses" of 0.5 to 1.0 γ/kg intravenously.

Results. The method has been used to measure heart force produced in the intact animal by cardiac glycosides(7), by hyperthermic agents(8), and by sympathomimetic amines(9). It is peculiarly suited to measure changes in heart force during the transitions of general(10) and spinal anesthesia(11), since it smoothly records the force of each contraction during the normal, conscious state as well as during anesthesia. Fig. 2 illustrates changes during induction and recovery from anesthesia with ether and, later in the same animal, with chloroform.

Summary. A strain gauge arch has been described which measures heart force changes in fully conscious, chronically operated dogs. Enclosure of the electrical component in a metal casing prevents insulation breakdown in body fluids.

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Received June 24, 1953. P.S.E.B.M., 1953, v84.

Effect of Antibiotic Dosage on Mortality from Whole Body X-Irradiation.* (20613)

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A large amount of data has accumulated on the effect of antibiotics on radiation injury. In general these drugs have been found to be slightly or moderately effective in reducing mortality. Varying amounts of antibiotics have been given at different intervals prior to and following radiation. This report is concerned with the effect of antibiotic dosage on the mortality from whole body x-irradiation.

Exp. 1. One hundred white male rats of the Wistar strain having a mean weight of 221 g, were separated randomly into 5 groups of 20 each. One untreated group served as a control while the 4 remaining groups received terramycin hydrochloride daily in amounts of 0.2 mg, 2.0 mg, 20 mg, or 200 mg, respectively. The antibiotic was given by stomach tube in a 2 cc aqueous solution beginning 72 hours and ending one to 4 hours prior to

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* This work performed under contract between the U. S. Atomic Energy Commission and Western Reserve University. Terramycin furnished by courtesy of Chas. Pfizer and Co., Brooklyn, N. Y.

TABLE I. Mortality and Survival Time in Control and Treated Animals. 5 groups, 20 rats in each group.

Dose of terramycin, HCl/rat/day	No. of 30-day survivors	% mortality	Mean survival time in days	s*	S.E.†
(mg)					
None	5	75	8.7	3.5	.9
.2	4	80	9.8	5.6	1.4
2	8	60	11.2	3.6	1.1
20	7	65	11.7	9.8	2.4
200	11	45	13.3	4.9	1.6

* s = Stand. dev. † S.E. = Stand. error.

x-irradiation, a total of 4 doses. No antibiotic was administered after radiation. Each rat received 660 r of whole body radiation. The housing of the animals and the method of administering the terramycin were similar to that previously described(1). The animals were weighed at intervals, 5 times during a 2-week period prior to the experimental period, and any rat whose weight gain was not satisfactory was discarded. The animals were observed daily for 30 days following x-irradiation. The physical factors of x-irradiation were as follows: 220 KV; 15 MA; mean target distance 32.1 cm; filters of 1 mm Al. and ½ mm Cu; output in air 124 r/min.; half-value layer 1.35 mm Cu.

Results. Mortality. In the control group 15 of 20 rats died during the 30-day experimental period (Table I). In the groups receiving 0.2; 2.0; and 200 mg/day of terramycin the number of deaths was 16, 12, 13, and 9, respectively, with mortalities of 80, 60, 65 and 45%, respectively. The χ^2 value for the difference between the 0.2 and 200 mg/day groups is 5.01 which is significant statistically. The χ^2 value for the difference between the control and the 200 mg/day terramycin group is 3.75. This approaches statistical significance (3.845) at the 5% level. The differences in mortality between the 2.0, 20 and 200 mg/day groups are not sufficient to reach statistical significance. However, there is a tendency for the mortality to decrease with increasing dosage of terramycin. *Survival.* The mean survival time for the control group was 8.7 days and for the groups receiving 0.2, 2, 20, and 200 mg/day of terramycin hydrochloride it was 9.8, 11.2, 11.7, and 13.3 days, respectively. Thus there appears to be a progressive increase in survival time with increasing dosage of terramycin. *Morbidity.* This was greater in the control group from the 4th to the 12th day and in the group receiving 200 mg terramycin from the 12th to the 30th day. In general, in the remaining groups the morbidity paralleled the mortality. Diarrhea was most marked in the group receiving the largest dose of terramycin. With decreasing amounts of terramycin the diarrhea was less marked but in none was it less than in the control group.

Exp. 2. Seventy-five white male rats of the C. F. Wistar strain with a mean weight of 211 g were divided randomly into 3 groups of 25 each. One untreated group served as a control while the 2 remaining groups received terramycin hydrochloride daily in amount of 20 and 200 mg per rat respectively. The schedule and mode of administration of the antibiotic were the same as for Exp. 1. No terramycin was administered after radiation. Each animal received 660 r of whole body radiation.

Results. Survival time and mortality for the 3 groups are given in Table II. In contrast to Exp. 1, there was no increase in survival time for the 20 mg/day group as compared to the control and only a slight increase for the 200 mg/day group. The difference in

TABLE II. Mortality and Survival Time in Control and Treated Animals. 3 groups, 25 rats in each.

Dose of terramycin, HCl/rat/day	No. of 30-day survivors	% mortality	Mean survival time in days
(mg)			
0	5	80	12.8
20	7	72	10.0
200	18	28	14.4

mortality rate between the control and the 20 mg/day treated groups was not significant. However, the differences between the control and the 200 mg/day groups and between the 20 and 200 mg/day groups were highly significant, *i.e.*, statistically at the 0.02 and 0.2% levels respectively. The highest dosage level of terramycin (200 mg/day) appears to have been definitely effective in lowering mortality. In this experiment diarrhea was only slightly more marked in the group receiving the largest dose of antibiotic than in the 20 mg/day and control groups.

Discussion. Koletsky and Christie(2) obtained a 31% reduction in mortality in rats given 120 mg/kg/day of streptomycin and 50,000 units/kg/day of penicillin intramuscularly, beginning as early as 10 days prior to and discontinuing 3 weeks after injection of lethal doses of P^{32}. Miller, Hammond, Tompkins, and Shorter(3) gave mice streptomycin subcutaneously from the 4th to the 28th day following 450 r whole body x-irradiation. Although the mice were not randomly distributed to study the effect of dosage, a review of their figures indicates that relatively large amounts of streptomycin were required for beneficial results. Thus 50 to 100 mg/kg/day of the antibiotic were ineffectual whereas 150 to 350 mg/kg/day reduced mortality from 37 to 55% in various groups.

Additional data pertinent to antibiotic dosage are available in the literature. In mice which received approximately 250 to 350 mg/kg/day of streptomycin subcutaneously, usually from the 4th to the 28th post-radiation day but in some instances from the day of x-radiation until the 24th post-radiation day, the mortality differences between control and treated groups ranged from 43 to 65%(4,5). The use of 40,000 units/kg/day of penicillin in conjunction with the streptomycin gave a reduction in mortality of from 30 to 41%, while subcutaneously administered chloramphenicol (about 100 mg/kg/day) reduced mortality by 24 and 25% in 2 groups of mice, respectively. Sodium salts of terramycin(5) and aureomycin (6) have also been effective in mice; when given subcutaneously in amounts of 25 and 50 mg/kg/day, respectively, mortality was lowered by 25 and 30%. However, Miller *et al.*(3) have concluded that chloramphenicol, aureomycin and terramycin are too irritating to be administered daily by subcutaneous injections, since necrosis of the skin occurred in many of the mice. These drugs were also given with the diet but dosage in these instances is difficult to evaluate.

Furth, Coulter and Howland(7) gave rats approximately 80 to 100 mg/kg/day of aureomycin or terramycin as part of the diet from the time of x-irradiation until the 28th post-radiation day. Aureomycin had no apparent effect on mortality but terramycin gave about an 18% reduction in mortality. A similar procedure in dogs which received 100 mg/kg/day of antibiotic orally, produced mortality reductions of 14 and 42% for aureomycin and terramycin, respectively(8,9). Smith, *et al.* (10) gave mice streptomycin sulfate in amounts of 75 to 1000 mg/kg/day, subcutaneously or orally, with somewhat variable but usually beneficial effect. In these experiments other undetermined factors influenced mortality to such a degree that evaluation of dosage was unsuccessful. Gustafson and Koletsky(1) gave rats approximately 1,000 mg/kg/day of terramycin hydrochloride by gavage for only 48 hours prior to x-irradiation and obtained a 24% reduction in mortality. No antibiotic treatment at all was given after x-irradiation.

In all reports in which antibiotics have been effective in reducing mortality in the x-irradiation syndrome the quantity administered has been relatively large. Amounts from 3 to 7 times the human dose on a comparable weight basis have been effective as against equivocal results for doses equal to or even twice the human dose. In this study amounts of terramycin approximately 30 times the corresponding human dosage were beneficial in reducing mortality. However, smaller doses were less effective and an amount comparable to the dosage required to stimulate growth in chicks and swine was ineffective. In our opinion the beneficial action of the terramycin was probably related to its antibacterial property. It seems likely that in the radiation syndrome the impairment of all normal defense mechanisms against disease makes it necessary to administer large amounts of antibiotic for maximum beneficial effect. In preliminary experiments a dose of 2000 mg/kg/day of terramycin (10 times the highest level used in this study) was tried but the preparation was so strongly acid that in many instances it caused perforation of the stomach and had to be abandoned(11).

Summary. Rats were given varying doses of terramycin hydrochloride beginning 72 hours and ending 1 to 4 hours prior to x-irradiation. The highest dosage level (200 mg/rat/day) was the most effective in lowering mortality. It is probable that larger doses of antibiotics are required in the x-irradiation syndrome than are required for the treatment of other diseases.

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Received June 26, 1953. P.S.E.B.M., 1953, v84.

Interference in Cortisone-Treated Hosts.* (20614)

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Cortisone has been shown to suppress inflammation(1) and antibody formation(2), to induce lymphocytolysis and dissolution of lymphoid tissue(3), to enhance viral multiplication(4), and to increase the severity of infections produced by a wide variety of organisms(5-8). It is evident that a multitude of effects may be attributed to the action of cortisone. The present paper reports the results of studies of the interference phenomenon in cortisone-treated hosts.

Materials and methods. Virus. An egg-adapted strain of influenza A (PR8) virus was used and prepared by allantoic inoculation of 11-day embryos with 0.05 ml of a 10^{-6} dilution of infected allantoic fluid; after incubation of eggs at 36°C for 42-48 hours, the fluids were collected and concentrated by the method of red cell adsorption and elution. A final concentration of 5% chicken red cells was used for adsorption of virus; after 45 minutes in the cold, the supernatant fluid was removed and the agglutinated cells resuspended in a small volume of 0.2 M phosphate buffer, pH 7.4. Elution of virus was carried out in a 37°C water bath for 4 hours. The red cells were sedimented by brief centrifugation and the supernatant fluid used as a source of virus after adjustment of the hemagglutinin titer to 2560.

Preparation of interfering virus. Virus prepared in the above manner was exposed to 5×10^{-3} M sulfur mustard, [bis (β-chloroethyl) sulfide], for 3 hours at room temperature. The viral suspension was then centrifuged briefly to remove precipitated material and the supernatant fluid used as an interfering suspension after testing for inactivation of virus. The advantages of a sulfur mustard-inactivated interfering suspension will be described elsewhere(9). *Tests of inactivation of virus.* 11-day embryos were inoculated allantoically with 0.5 or 0.05 ml of undiluted suspension, and 0.05 ml of serial 10-fold dilutions

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* This work was aided by a grant from the Research Committee of the University of California.

TABLE I. Results of Interference Tests in Cortisone-Treated Eggs.

Pretreatment of host (ml)	Interfering virus (AD)*	Challenge virus (ID$_{50}$)	Individual HA titers	Geom. mean titers†
.2 water	512	400	<10, 10, 20, 20, 20, 20	<16
"	256	"	<10, <10, 10, 10, 20, 20	<13
"	128	"	10, 20, 20, 40, 80, 80	32
.2 cortisone	512	"	10, 20, 20, 20, 20	17
"	256	"	40, 80, 160, 160, 160, 320	127
"	128	"	80, 160, 160, 320, 320, 640	226
.2 cortone vehicle	256	"	10, 20, 20, 20, 20, 40	20
.2 water	0	"	1280, 1280, 1280, 1280, 1280, 2560	1437

Time between each inj. = 30 min. (at 36°C).

* Refers to No. of agglutinating doses of interfering virus injected into eggs.

† Individual hemagglutinin titers of less than 10 were arbitrarily taken as 10 in calculation of geometric mean titers.

of virus. Four eggs were used for each dilution. After incubation of embryos for 42-48 hours at 36°C, the individual fluids were tested for hemagglutinins by mixing 0.1 ml of fluid with 0.25 ml of 1% cells. The test was read by the pattern method(10) after 30 minutes at room temperature.

Infectivity titrations of challenge virus. Serial 10-fold dilutions of virus were made in beef-heart infusion broth and 0.05 ml of dilution inoculated allantoically into each of four 11-day embryos. After the usual incubation, the fluids were tested for development of hemagglutinins as described above. The ID$_{50}$ was calculated by the method of Reed and Muench(11). *Interference tests.* Groups of six 11-day embryos were inoculated allantoically with varying amounts of inactivated virus; after 30 minutes at 36°C, a challenge dose of active homologous virus was given. The individual allantoic fluids of live embryos (following incubation at 36°C for 42-48 hours) were collected and titrated for their content of hemagglutinins. Chemical treatment of the host consisted of allantoic injections of commercial cortisone acetate (Merck: 25 mg/cc) given 30 minutes before introduction of interfering virus into the host. Controls were injected in a similar manner with the cortone vehicle (0.9% benzyl alcohol, 0.9% sodium chloride, 0.4% polyoxyethylene sorbitan monooleate, 0.5% sodium carboxymethylcellulose, distilled water, q. s. ad 100%). *Hemagglutinin titration.* Serial 2-fold dilutions of virus were made in saline and 0.5 ml of viral dilution was mixed with 0.5 ml of 1% chicken red cells. The test was read after 30 minutes at room temperature by the pattern method. The final dilution of virus in the last tube showing complete agglutination was taken as the end point, and the titer expressed as the reciprocal of the dilution of virus in this tube.

Results. Increased yield of virus in cortisone-treated eggs receiving minimal completely interfering doses of inactivated virus. The results of interference tests in untreated and cortisone-treated embryos are shown in Table I. In the interpretation of results, hemagglutinin titers of 20 or less were accepted as complete interference with propagation of challenge virus since titers of residual interfering virus may equal but seldom exceed this value.

It is evident that introduction of 5 mg of cortisone into eggs 30 minutes before injection of interfering virus resulted in higher yields of viral hemagglutinins. Comparison of the mean hemagglutinin titers of control and experimental series given 400 ID$_{50}$ of challenge virus 30 minutes after interfering virus revealed 7- to 10-fold higher titers of virus in cortisone-treated eggs. This effect was noticeable, however, only when a minimal completely interfering dose (256 AD in the experiment shown in Table I) or less of inactivated virus was employed. Despite prior injections of cortisone, an increased yield of virus was not demonstrable in eggs receiving 512 agglutinating doses of interfering virus.

Similar findings were obtained in other tests using 40,000 ID$_{50}$ of challenge virus (results not shown); while injections of this amount of challenge virus may result in higher mean titers of hemagglutinins in both control and experimental eggs, the increase in yield of virus in cortisone-treated hosts was approximately of the same order of magnitude as that observed with 400 ID_{50} of challenge virus.

The effect produced by injections of cortisone was not ascribable to the action of substances present in the cortone vehicle, for prior treatment of embryos with 0.2 ml of the latter failed to cause any significant increase in final yield of viral hemagglutinins.

In other interference tests in which 11-day embryos were given 2.5, 5 and 7.5 mg of cortisone 30 minutes before interfering virus, it was found that all 3 concentrations of cortisone were equally effective in promoting an increased yield of viral hemagglutinins (results not shown) in the presence of a minimal completely interfering dose of inactivated virus. This was attested by the more or less identical yields of viral hemagglutinins in all three groups of cortisone-treated eggs.

Discussion and summary: The results reported herein have shown that prior injections of cortisone in embryonated eggs subsequently given minimal completely interfering doses of inactivated virus followed by challenge virus resulted in yields of viral hemagglutinins which were 7- to 10-fold greater than that found in untreated eggs. All 3 concentrations of cortisone (2.5, 5 and 7.5 mg) utilized in these experiments appeared to be equally effective in this respect.

An enhanced growth of influenza viruses in cortisone-treated eggs has been described previously(4), and the viral concentration of pooled allantoic fluids from cortisone-treated eggs was shown to be 123 to 155% of the viral concentration of control eggs (for influenza A virus). In the interference experiments reported in this paper, the yield of virus in cortisone-treated eggs was 700 to 1000% of the yield in untreated embryos. In view of this discrepancy in the increased yields of virus in the two reports, it would appear that enhancement of reproduction of challenge virus in residual free cells not blocked by interfering virus was not the sole determining factor in the increased yield of virus reported in this paper.

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Received July 1, 1953. P.S.E.B.M., 1953, v84.

Histopathology of Amino Acid Deficiencies. II. Threonine.* (20615)

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The unfavorable effect of protein deficiency on body economy has been frequently described. Whether or not there is a specificity of tissue response to protein inadequacy, or more particularly to specific amino acid deficiency, is not well established. A previous report of phenylalanine deficiency(1) described alteration of the anterior pituitary, gonads, accessory sex glands, bone and adrenal glands. Investigations of threonine reported

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* This investigation was supported by a grant from the National Institutes of Health.

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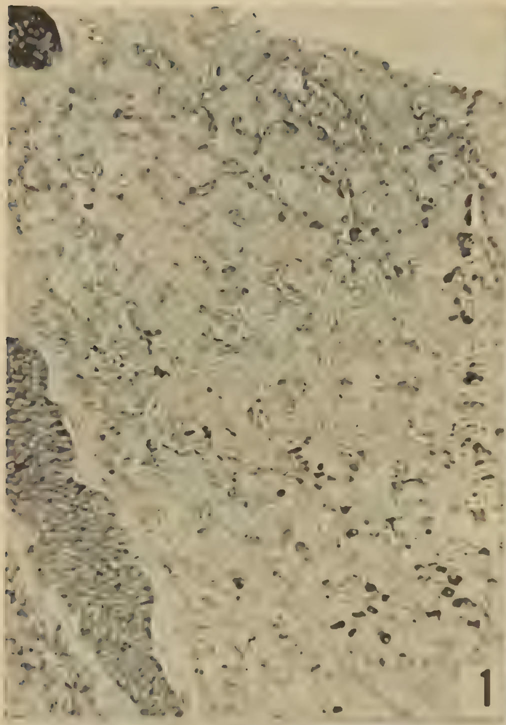
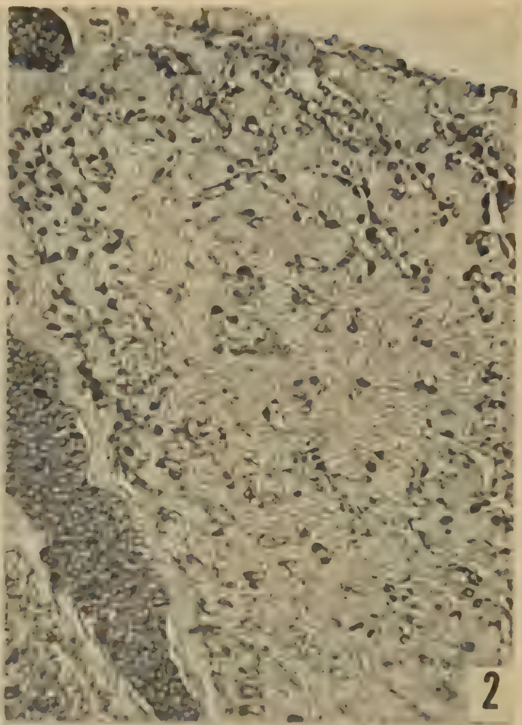
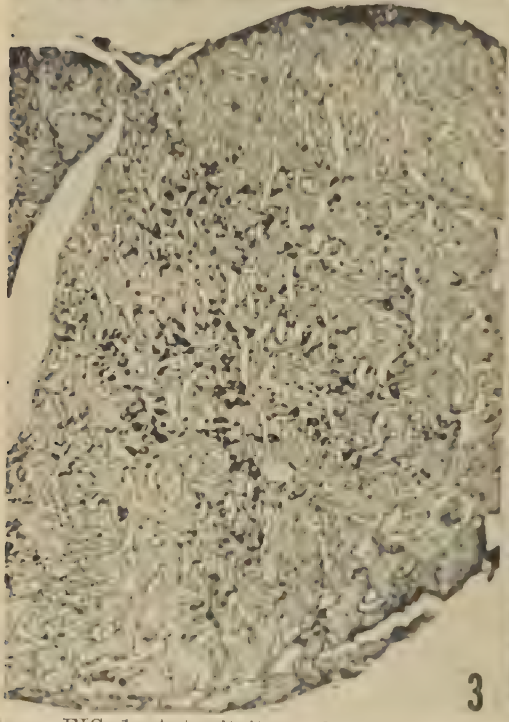






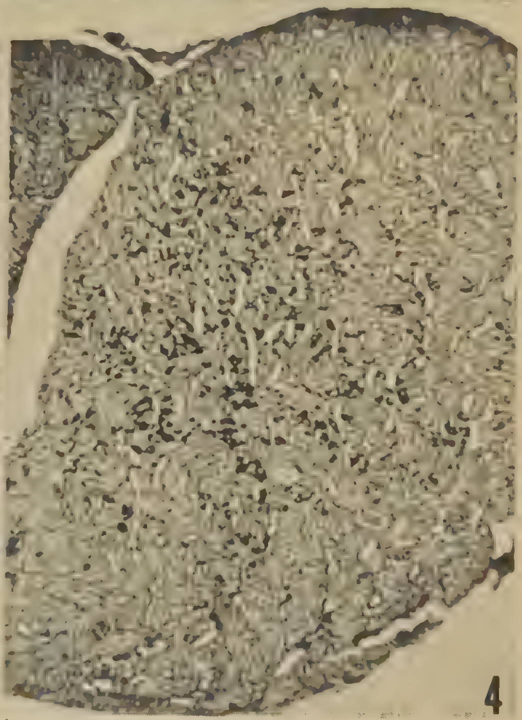


FIG. 1. Ant. pituitary of a control rat stained by Gomori method. Gomori positive cells (dark) are the thyrotropic basophils. ×100.

FIG. 2. Same section and field shown in Fig. 1 restained by periodic acid-Schiff (PAS) method. All basophils are PAS positive. Visual subtraction of Gomori positive thyrotropic cells indicates the gonadotropic basophils. ×100.

FIG. 3. Ant. pituitary of a threonine-deficient rat stained by Gomori method. Gomori positive cells are conspicuous. ×100.

FIG. 4. Same section and field shown in Fig. 3 restained by the PAS method. Only those cells which were Gomori positive in Fig. 3 are PAS positive in this photomicrograph demonstrating the absence of gonadotropic basophils. ×100.

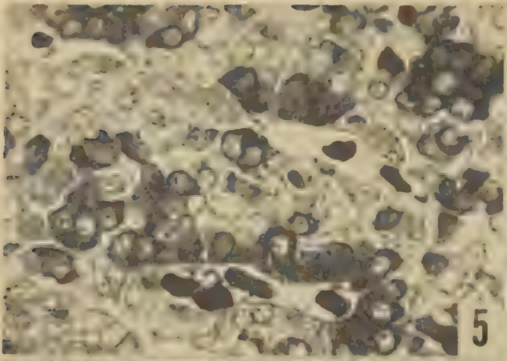
in the literature deal principally with the requirement of this amino acid and not with histological effects of its deficiency. Nasset, Anderson and Siliciano(2), in a study of nitrogen balance in adult rats fed a low threonine diet, reported that death, in many instances, resulted from edema of the esophagus. Bauer and Berg(3) described ascites and generalized edema in growing mice maintained on a threonine deficient diet.

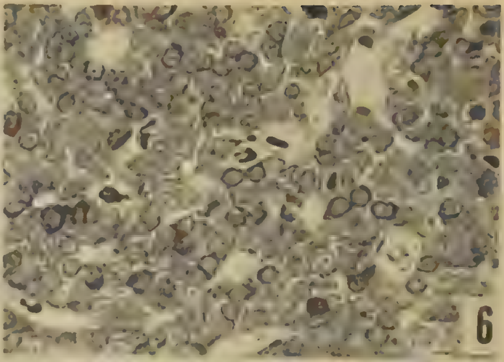
Materials and methods. Sprague-Dawley male rats, averaging 100 g weight, were divided into 3 groups. One group of 5 received a complete "synthetic" diet containing 19 amino acids as described by Rose, Oesterling and Womack(4). Another group of 12 received the same diet except that this diet was completely lacking in threonine. These diets were fed *ad libitum*. A third group of 9, referred to as "starved control", were fed a limited amount of the control diet on alternate days. All rats were weighed 3 times weekly. After 45 days the rats were sacrificed by exsanguination under ether anesthesia. Two of the deficient and one of the "starved control" rats, however, were not sacrificed at this time but were placed on a complete recovery diet for 45 days and then sacrificed. The tissues removed for histological examination, except the pituitaries, were fixed in Bouin's fluid and stained with hematoxylin and eosin. The pituitaries were fixed in Zenker-formol and stained by methods described below. For demonstration of the eosinophilic cells of the anterior pituitary, sections were stained with acid fuchsin. Other sections were stained by methods described by Purves and Griesbach(5,6) to identify and differentiate thyrotropic and gonadotropic basophils of the anterior pituitary. In using these procedures it was necessary first to treat the sections by a method originated by Gomori(7) and developed by Halmi(8) and to photograph these sections. The same sections were then restained by the periodic acid-Schiff (PAS) reaction, which stained all basophils, and the identical fields rephotographed. In this manner records of two different technics could be obtained for the same section and field. By comparing the two photographs it was possible to visually subtract the Gomori positive cells from the PAS positive cells and thus identify the presence or absence of both thyrotropic and gonadotropic basophils (Fig. 1, 2, 3, 4).

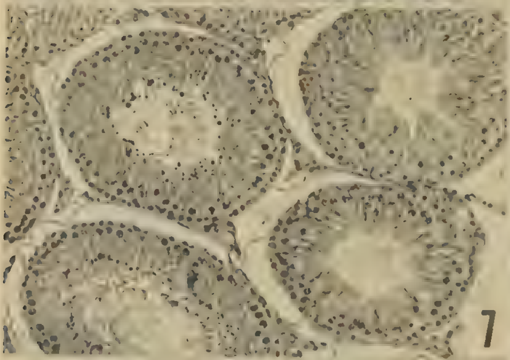
Observations. Loss in body weight, an unkempt appearance and other general deficiency symptoms developed in those rats deprived of threonine. The average loss in body weight was 41(±9) g for each of the threonine deficient rats and 11(±6) g for each of the "starved control" rats. Each control rat gained an average of 131(±11) g. The deficient rats which had been placed on the recovery diet gained weight rapidly and, at the termination of the recovery period, weighed as much as the average control rat.

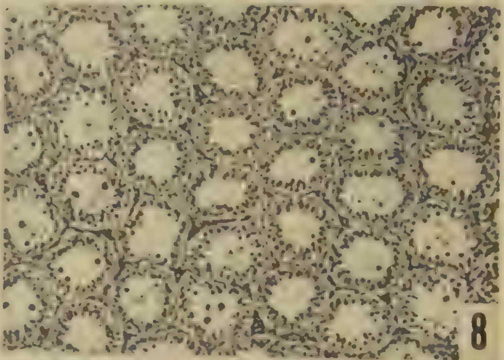
At autopsy the adrenals, testes and pituitaries were weighed and, although these organs in the deficient rats weighed considerably less than normal, the weights were variable and appeared to have no correlation with the loss in body weight. Ascites was present in only one rat but the testes of all deficient rats were soft and flabby to the touch. In general, all organs, when examined grossly, were smaller than normal. Those tissues in which histological change was observed are reported below. In all other tissues examined histologically, alterations, if they occurred, were not of an order which could be detected by the methods employed.

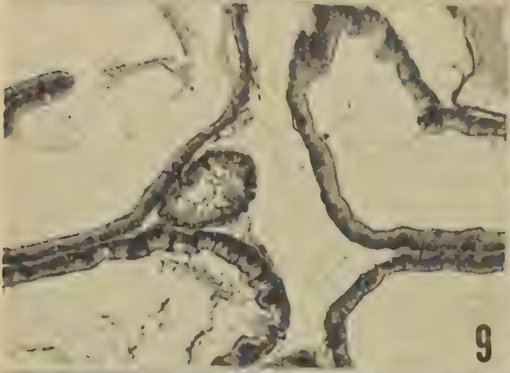
The testes of the threonine-deficient rats were extremely atrophic with no evidence of active spermatogenesis (Fig. 8). Some resting spermatogonia were in evidence, but the tubules were composed principally of Sertoli cells resting on the basement membrane. Single nucleated and multinucleated degenerating cells were commonly observed in the lumen. The testicular interstitial tissue cells were smaller and flatter than normal with little cytoplasm. The epididymides, seminal vesi-

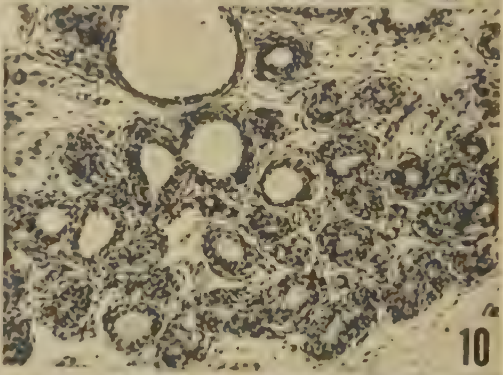


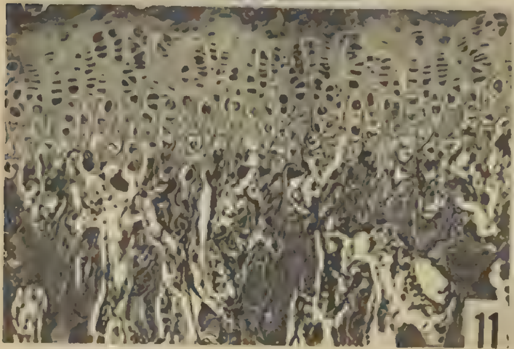












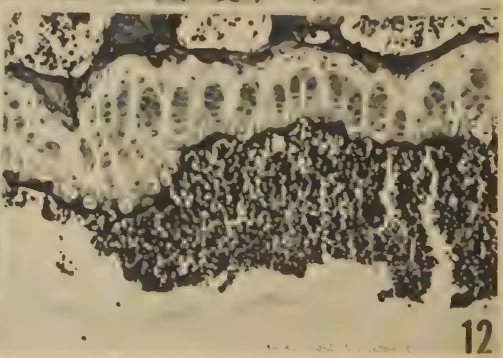


FIG. 5. Ant. pituitary of a control rat stained with acid fuchsin. Eosinophils are prominent. ×470.

FIG. 6. Ant. pituitary of a threonine-deficient rat stained with acid fuchsin. Eosinophils are fewer, smaller and fainter. ×470.

FIG. 7. Testes of a control rat. ×100.

FIG. 8. Testes of a threonine-deficient rat. ×100.
FIG. 9. Ventral prostate of a control rat. ×100.
FIG. 10. Ventral prostate of a threonine-deficient rat. ×100.
FIG. 11. Epiphyseal plate of tibia of a control rat. ×100.
FIG. 12. Epiphyseal plate of a threonine-deficient rat. ×100.

cles and ventral prostates were in an extremely atrophic condition. The epididymal epithelium was considerably lower than normal and the cells crowded. Free cells, sloughed from the seminiferous tubules, had moved into the lumen of the epididymis. The seminal vesicles and prostates were in a state similar to that observed in these organs following castration. The prostatic acini were much smaller than normal and the acinar cells extremely regressed (Fig. 10). The negative image of the Golgi apparatus, which could plainly be observed in the normal prostate (Fig. 9) and is considered a criterion of the prostatic secretory activity, could not be observed in the deficient prostatic epithelium. Coincident with acinar regression, there occurred an increase in prostatic interstitial tissue. The testes of the "starved control" rats were smaller than normal with reduction in the size of the seminiferous tubules and partial interruption of spermatogenesis. The Leydig cells were morphologically normal. That the capacity of the testicular interstitial tissue to secrete its hormone was reduced was made evident by the histological appearance of the ventral prostate. This organ appeared to be partially stimulated, since its acini were large and composed of both tall secretory and flat non-secretory cells.

The thyroid glands of the threonine-deficient rats were reduced in size but the height of the follicular cells did not appreciably differ from the normal. The follicles were variable in size ranging from large peripheral colloid filled follicles to small centrally located follicles, with and without colloid. The thyroids of the "starved control" rats were indistinguishable from the normal in size and histology.

The thymus gland was very prominent in the control rats but not identifiable grossly in the threonine-deficient rats. Partial thymic involution occurred in the "starved control" rats but the cortical and medullary areas remained histologically intact. No extensive increase in the connective tissue of this organ was observed.

The anterior lobe of the hypophysis was the only portion of this gland histologically affected by the lack of threonine. The eosinophilic cells were conspicuously reduced in number, size and staining capacity (Fig. 6). These cells had only a thin ring of lightly staining cytoplasm. The thyrotropic basophils, demonstrated as being Gomori positive, were present in numbers and morphologically unaltered (Fig. 3). Other basophils, which in the normal rat are PAS positive (Fig. 2), were not demonstrable in the deficient rats after use of the PAS method (Fig. 4). The anterior pituitary chromophilic cells of the "starved control" rats compared favorably with those of the control rats in size and distribution.

Bone growth was considerably reduced in the threonine-deficient rats. The tibial epiphyseal cartilage plate was thinner than normal. No bony trabeculae were present in the diaphysis (Fig. 12). The bone shaft was also thinner and its circumference less than normal.

The rats placed on the recovery diet made uneventful complete recoveries from the deficiency. Histological examination of their tissues revealed that they were normal in all respects.

Discussion. Comparison of the effects of partial inanition ("starved control") with those of threonine deficiency demonstrated that tissue regression was more pronounced in rats fed the threonine-deficient diet. Siperstein(9) described testicular damage resulting from chronic and acute inanition. Maun, Cahill and Davis(10,11), Adamstone and Spector(12) and Schwartz, Scott and Ferguson (1) reported similar damage following specific amino acid deficiencies. Jackson(13) and Mulinos and Pomerantz(14) described thymic involution in studies of chronic and complete inanition. Dougherty and White(15) and Frank, Kumagai and Dougherty(16) reported that adrenal cortical hormones diminish lymphatic tissue by destruction of lymphocytes. It is our opinion that the testicular damage and thymic involution of the threonine-deficient rats resulted from the lack of this amino acid superimposed upon the effects of partial inanition. Since the adrenals gave no evidence of being hypersecretory, it was unlikely that thymic degeneration resulted from stimulation of the adrenal glands.

The assumption that a primary effect of the lack of dietary threonine was expressed upon the anterior pituitary appears to be justified in the present study. Purves and Griesbach (5) reported that all basophils of the anterior pituitary react in a positive manner to the PAS reaction. These authors later described (6) the identification of thyrotropic cells by use of the Gomori method. By employment of the methods they described, it was evident that a total lack of threonine, while depressing the gonadotropic basophils, had no effect upon the thyrotropic cells. Comparison of Fig. 1 and 2 demonstrates the presence of the thyrotropic and gonadotropic cells in the normal pituitary. Comparison of Fig. 3 and 4 reveals that in the threonine-deficient rat gonadotropic basophils were not in evidence but the thyrotropic cells were intact. The lack of gonadotropic hormone resulting from this diminution of gonadotropic cells resulted in atrophy of the testicular tubules and interstitial tissue. The nonfunctional state of the Leydig cells was reflected by the atrophy of the accessory sex glands. Further interference with the pituitary gland was evidenced by alteration of the eosinophilic cells. The defective appearance of the tibial shaft and epiphyseal plate are an expression of the impediment of somatic growth.

Since no serious alteration in the chromophilic cells of the anterior pituitary was observed in the "starved control" rats, it is reasonable to assume that the lack of threonine resulted in the pituitary changes described above.

The adrenals of the threonine-deficient rats were small but no change in the regions of the cortex and medulla was apparent. Since the pituitary eosinophils were reduced considerably in size and number and since the PAS positive gonadotropic basophils were completely lacking, is it possible that the adrenal cortical stimulating hormone has its source in those cells referred to as thyrotropic cells?

Despite the marked changes brought about by the complete lack of threonine, all rats placed on the recovery diet made complete recoveries. Thus, any deterioration resulting from threonine deficiency is reversible.

Summary. Young male rats fed a "synthetic" diet, complete in every respect except for the total lack of threonine, showed marked loss in weight, interruption of somatic growth, atrophy of the testes and accessory structures and alterations in the chromophilic cells of the anterior pituitary. The deficiency effects were reversed by adding the missing amino acid to the diet.

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Received July 3, 1953. P.S.E.B.M., 1953, v84.

Phosphocreatine Content of Mammalian Cardiac Muscle.* (20616)

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The values reported in the literature for phosphocreatine in mammalian cardiac muscle vary widely. For the intact rat heart the published figures, expressed in terms of phosphocreatine-P, vary from 5.7 to 12 mg % (1-4). For the intact guinea pig heart and cat heart, the figures are 5.2 and 6.0 mg %, respectively(5,6). For the intact dog heart, the figures vary from 4.2 to 9.0 mg %(6-8). For the isolated dog heart (heart-lung preparation) the published values range from 6.2 to 21.5 mg %, depending on the experimental conditions(8-10). Wollenberger(8) and Pollack *et al.*(9) state that the phosphocreatine content of the heart in the intact animal is considerably below that of the normal heart-lung preparation. Mommaerts(11) states, without citing references, that cardiac muscle may contain one-tenth as much phosphocreatine as striated muscle, and he gives the value 2 x 10^{-6} mole per g for cardiac muscle, *viz.* 6.2 mg % phosphocreatine-P.

In connection with our studies on the effect of drugs on the metabolism and performance of the isolated dog heart (heart-lung preparation), we found it necessary to redetermine the "resting" values of phosphocreatine in mammalian heart muscle. At the same time we have made determinations of the corresponding values for the labile "nucleotide" phosphorus.

Methods. Pentobarbital anesthesia was used in all animals including those designated for heart-lung preparations. Artificial respiration was applied before opening the chest. Heart-lung preparations were performed by the conventional procedure except that the blood donors were anesthetized with chloroform. A sample of heart muscle was cut from the left ventricle and quickly immersed in liquid air or in a mixture of CO_2-snow and ether (temp. —60°C). When liquid air was used the frozen tissue was ground into a fine powder in a mortar chilled by adding successive portions of liquid air to make sure that the tissue did not thaw. The powdered material was then transferred to a previously weighed Erlenmeyer flask containing 5% trichloracetic acid using a spoon and a wide-necked funnel that had been dipped in liquid air. The flask was shaken to mix the contents and then reweighed. The volume of 5% trichloracetic acid employed was adjusted to give a dilution of 1:10, reckoning the water content of the tissue as 80%. Not more than 10 minutes was allowed to elapse between the fixing of the powder with acid and the time when the protein-free filtrate was neutralized. During this period of time all operations were carried out at a temperature which did not exceed 2°C. Inorganic phosphate and phosphocreatine were determined by the method of Fiske and Subbarow(12) and the labile "nucleotide" phosphorus was calculated by subtracting the sum of the phosphocreatine and inorganic phosphorus from the value for the total inorganic phosphorus obtained after hydrolysis for 10 minutes in 1 N HCl at 100°C. In a subsequent publication we are discussing the extent to which labile "nucleotide" phosphorus represents adenosine triphosphate. Equally satisfactory results were obtained by the use of the more available CO_2-snow. After the tissue was fixed in a CO_2-snow-ether mixture it was dried with filter paper and weighed quickly in a closed weighing bottle. It was then ground in a mortar with sufficient CO_2-snow to prevent thawing. Towards the end of the grinding, a small amount of coarsely ground glass was added to ensure fine pulverization. The powder was then transferred quantitatively into an Erlenmeyer flask containing the calculated amount of cold 5% trichloracetic acid.

Results. It can be seen from Table I that the phosphocreatine values for intact dog hearts obtained with the CO_2-snow procedure

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* This study has been supported by a grant from the American Heart Assn.

TABLE I. High-Energy Phosphate Content of Intact Mammalian Hearts. Heart tissue removed under pentobarbital anesthesia with artificial respiration. All values are expressed as mg phosphorus per 100 g fresh tissue.

Species	No. of animals	Method of freezing	Phosphocreatine-P, mean ± S.E.	Inorganic-P, mean ± S.E.	Labile ''nucleotide''-P, mean ± S.E.
Dog	19	CO_2-snow	18.2 ± .88	19.64 ± .68	37.1 ± .7
"	18	Liquid air	19.9 ± .84	20.14 ± .72	39.5 ± .98
Cat	6	"	15.04 ± 1.45	18.13 ± 1.34	34.3 ± .62
Rabbit	5	"	14.23 ± 1.66	19.32 ± 1.17	33.9 ± 1.2
Rat	6	"	9.0 ± .0	23.6 ± .33	29.9 ± .97

TABLE II. High-Energy Phosphate Values on Same Sample of Frozen Dog Ventricle Obtained by Two Methods: One involves grinding the frozen tissue with liquid air or CO_2-snow prior to treatment with trichloracetic acid, the other omits the grinding with liquid air or CO_2-snow.

Method	No. of animals	Phosphocreatine-P, mean ± S.E.	Inorganic-P, mean ± S.E.	Labile ''nucleotide''-P, mean ± S.E.
		Intact hearts		
Liquid air	11	18.14 ± 1.3	19.62 ± .97	37.37 ± .68
Trichloracetic acid	"	10.26 ± .89	27.88 ± .79	38.6 ± .65
		Failing isolated hearts		
Liquid air	6	25.23 ± 2.13	16.0 ± .11	37.81 ± .82
Trichloracetic acid	"	15.56 ± 1.78	25.68 ± 1.21	38.0 ± .14

are not significantly lower, as might possibly be expected, when compared with those obtained by using liquid air. It will also be noted that these values are higher than those reported by previous authors, being over 3 times as high as those of Cruickshank *et al.* (6) and twice as high as those of Pollack *et al.* (7) and of Wollenberger(8). Although the procedures used by these 2 latter authors included artificial respiration and also ensured quick freezing of the heart tissue, Pollack *et al.* put the frozen tissue through a meat grinder before extracting with acid, and Wollenberger ground the frozen tissue in ice-cold acid. It is quite possible that in both procedures the tissues were permitted to thaw before the trichloracetic acid had had time to diffuse thoroughly through the tissue and stop the enzymatic hydrolysis of the labile phosphocreatine. To test this type of procedure we carried out the experiment indicated in Table II. In this experiment a piece of frozen tissue was broken in two, one was powdered with liquid air or CO_2-snow as described above and the other was ground in ice-cold trichloracetic acid. The average phosphocreatine value of the 11 intact hearts was 18.1 mg %, as determined by our standard technic as compared with the value 10.3 mg % obtained when the frozen tissue was ground directly with ice-cold trichloracetic acid (Table II). Equally sharp differences were obtained with failing hearts: 25.2 mg % as compared with 15.6 mg %. It is thus clear that in dealing with labile compounds like phosphocreatine, the tissues should be powdered in the frozen state before extraction with acid.

Table III shows that the phosphocreatine content of the non-failing isolated dog heart is not higher than that of the normal heart in the intact animal. The lower results obtained by Pollack *et al.*(7) and Wollenberger(8) for the normal intact hearts as compared with the values they obtained for the isolated hearts cannot be explained by suggesting that the phosphocreatine in the intact heart is enzymatically more labile than in the isolated heart. It is seen from Table II that the phosphocreatine value of the failing isolated heart obtained by using their procedure (15.6 mg %) is significantly lower (Table II) than those obtained by using the standard procedure (25.2 mg %). It is possible, however, that in Wollenberger's experiments, the isolated hearts may have undergone some degree of failure because of the longer period of 82

TABLE III. High-Energy Phosphate Content of Normal and Failing Isolated Dog Hearts (Heart-Lung Preparations). Values are expressed as mg phosphorus per 100 g fresh tissue.

Time of survival in min.	Heart rate		Systemic output in ml/min.		Serum glucose, mg %	Phosphocreatine	Inorganic-P	Labile "nucleotide"-P
	Initial	Final	Initial	Final				
					Normal hearts			
30	192	192	744	708	20	14.6	20.1	35.5
"	168	164	684	624	74	21.6	18.3	35.1
"	144	140	792	888	41	18.2	13.1	33.2
"	156	140	804	708	66	20.9	13.1	38.8
"	176	164	552	552	27	20.5	20.4	30.1
"	144	144	780	732	223	24.5	13.7	33.2
"	156	120	720	792	50	13.7	19.3	31.8
				Mean ± S.E.		19.15 ± 1.47	16.86 ± 1.29	33.9 ± 1.07
				Failing hearts (pentobarbital)				
75	156	128	456	300	—	20.4	21.4	—
53	180	124	912	180	44	20.5	15.9	30.2
65	220	148	708	276	75	30.9	12.2	38.8
40	120	128	660	324	67	23.6	15.5	38.4
52	120	124	720	216	60	27.4	16.3	37.4
50	164	128	828	240	106	23.0	16.9	38.5
30	140	116	792	84	111	29.8	17.2	36.9
80	160	124	528	228	70	16.7	17.8	36.9
26	140	104	—	10	—	24.3	11.9	37.0
				Mean ± S.E.		23.5 ± 1.58	16.9 ± .99	37.8 ± 1.05

minutes which elapsed in his experiments after the heart was isolated. An indication of this is given by the fact that the average systemic output in his experiments was 413 ml, whereas in ours it was 711 ml, although the temperature of the blood, the venous inflow level, and the artificial resistance were not significantly different in both sets of experiments. However, the resistance of the venous inflow tube may have been different: in our case the tube was 8 cm long with an inner diameter of 0.6 cm.

Table III also shows that in barbiturate-induced failure the phosphocreatine content of the heart-lung preparation is higher than that of the intact heart. This finding is in harmony with that of Wollenberger, although the difference is not as striking as in his results. This constitutes further evidence that in barbiturate failure which is known to respond to digitalis, there is probably an interference with energy utilization rather than with energy production(13). It should be mentioned, however, that Greiner(14), using a different preparation, obtained results which indicate that the oxygenated, severely hypodynamic papillary muscle of the cat is characterized by a decrease in adenosine triphosphate content, and that digitalis treatment not only corrects the muscle failure but also restores the adenosine triphosphate level to normal.

Summary. 1. "Resting" values for the phosphocreatine content of the ventricles of several mammalian species are reported and an explanation is offered for some of the low values recorded in the literature. The necessary precautions are noted if maximal values are to be obtained. 2. The isolated dog heart (heart-lung preparation) does not contain more phosphocreatine or adenosine triphosphate than the intact heart provided that heart failure has not set in. If, on the other hand, failure is induced by barbiturates the level of phosphocreatine increases without a change in the labile "nucleotide" phosphorus. This constitutes further evidence that this kind of heart failure is not due to the lack of production of high-energy phosphate bonds.

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Received July 3, 1953. P.S.E.B.M., 1953, v84.

Electrocardiograms and Serum Electrolyte Levels in Hamsters with Adrenal Insufficiency and Hypercorticalism.* (20617)

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In previous reports, Wyman, Fulton, and Shulman have described the circulatory changes in the transillumined cheek pouch of the hamster, (*Mesocricetus auratus*) after adrenalectomy and during treatment of the normal animal with cortisone(1). Vasoconstriction occurred consistently during cortisone administration. Furthermore, occasional vasomotion, frequent vasoconstriction, bradycardia, and slow blood flow occurred prior to death from adrenalectomy. Zweifach and Chambers, however, described a loss of vascular tone and absence of vasomotion in the mesoappendix of the adrenalectomized rat(2). In view of the evidence that hyperpotassemia may stimulate smooth muscle(3,4), a possible explanation for the discrepancy might be significant differences in serum potassium levels in the 2 species after adrenalectomy. Snyder and Wyman(5) reported a rise in the serum potassium of over 100% in the adrenalectomized hamster as compared with reported elevations of less than 50% in the adrenalectomized rat. In order to test the possible relationship between vasoconstriction, cardiac changes, and serum electrolyte levels, sodium and potassium determinations were made and electrocardiograms taken for each day following adrenalectomy and every other day during cortisone administration.

Materials and methods. Twenty-four male hamsters, 80 to 100 g in weight, were adrenalectomized using a dorsolateral approach in a single operation. Purina Laboratory Chow and tap water were given *ad libitum* and the hamsters received the usual postoperative care. The 24 hamsters were divided into 3 groups of 8 animals each, and on every third day an electrocardiogram was taken and blood was drawn by heart puncture for electrolyte determinations on each group so as to provide data for each postoperative day for their period of survival. Three groups of 8 intact hamsters were similarly used for cortisone studies except the electrocardiograms and heart punctures were made every other day throughout the entire period. Each animal was given a daily injection of 5 mg cortisone acetate (Cortone, Merck)‡. *Electrocardiograms* were obtained by means of hypodermic

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* This investigation was supported in part by a research grant from the National Heart Institute of the National Institutes of Health, Public Health Service.

† Messrs. Benotti of the Boston Medical Laboratory made the electrolyte determinations. The work has benefitted immeasurably by numerous conferences with Dr. Maurice H. Shulman. We are indebted to Dr. David Littman for advice in interpreting the electrocardiograms, to Mr. Frederick W. Maynard for technical photographic advice, and to Miss Lois L. Smith and Miss Roma Kagan for valuable assistance.

‡ Kindly supplied by Dr. Elmer Alpert, Merck and Co.

TABLE I. Adrenalectomized Hamsters; ECG, Serum Na and K.

Days after adrenalectomy	No. used and surviving	mEq/liter		Millivolts	
		Na	K	T wave	P wave
–3*	24	145 (137–153)†	5.97 (5 – 8)	.33 (.31–.36)	.20 (.18–.22)
1	24	137.6 (135–141)	7.1 (6.7– 8)	.20 (.16–.24)	.12 (.11–.13)
2	22	137.2 (134–141)	10.1 (6.5–12.5)	.25 (.19–.31)	.15 (.12–.18)
3	16	121.5 (95–140)	9.0 (7.7–11.2)	.16 (.00–.20)	.09 (.05–.10)
4	11	—	9.2 (7.7–11.8)	.19 (.16–.21)	.09 (.07–.11)
5	7	132.7 (123–139)	10.1 (7.3–15+)	.23 (.19–.27)	.10 (.07–.13)
6	0				

* Three day preadrenalectomy values for 24 hamsters in the group. After adrenalectomy, ECGs and heart punctures were made on 8 hamsters every third day.

† In this and in Table II the figure is the avg; figures in parentheses are the range.

electrodes, a preamplifier (Grass Instrument Co.), and a cathode ray oscilloscope, and were recorded on linograph paper in a kymograph camera (Grass Instrument Co.). Unipolar precordial leads VC-5 and VL were employed. Anesthesia was 10 mg per 100 g body weight sodium pentobarbital (Nembutal, Abbott). Serum for the sodium and potassium determinations was obtained from centrifuged blood taken directly from the heart with hypodermic syringes. The blood serum samples were prepared as follows: 0.1 ml of the serum was pipetted into a 25 ml volumetric flask containing lithium chloride solution, 5000 p.p.m. (0.75 ml for K, 4 ml for Na), and diluted to 25 ml with distilled water. Electrolytic determinations were made on a flame photometer (Barclay) and are reported in mEq/liter. A *control group* of 5 hamsters was used to determine the effect on the electrocardiogram of 5 successive heart punctures at 3-day intervals. Serum sodium and potassium were also determined for this group.

Results. After 5 successive heart punctures, the electrocardiogram showed no significant alterations in the control series. The average electrolyte levels on each successive heart puncture were found to be about 145 mEq/liter for sodium and 7 mEq/liter for potassium. These values did not vary significantly on each successive heart puncture and are similar to those reported by Snyder and Wyman(5). The slight differences in absolute values may be attributed to the manner in which the blood was obtained.

Adrenalectomized hamsters gradually lost weight and lived for an average of 5 days. The changes in the electrocardiogram and the serum electrolytes are shown in Table I and Fig. 1 and 2 A, B, C. The most significant electrocardiographic changes were a lowering of the P and T waves accompanied by bradycardia and, in some cases, a prolonged QT interval. The serum electrolyte levels exhibited a rise in potassium and a fall in sodium. It was noted that as the serum potassium rose, the T wave became shallower. In some cases where the potassium level exceeded 15 mEq/liter, a complete reversal of the T wave was observed (Fig. 1).

Cortisone-treated animals also lost weight but lived an average of 16 days. During the entire study the electrocardiogram showed no significant changes related to the electrolyte levels (Table II; Fig. 2D, E, F). This was true even in the stages of treatment where there was marked vasoconstriction in the hamster cheek pouch (10 to 12 days). The two high values for the T and P wave voltages on the twelfth and sixteenth days were correlated chronologically with the appearance and persistence of the vasoconstriction. The values were normal, however, on the intervening fourteenth day. Serum electrolyte levels remained essentially normal, although the potassium values were on the low side of the normal range during the treatment.

Discussion. The electrocardiographic changes reported in this paper are comparable with those reported for severe adrenal insufficiency in dogs(6). It is well established that the sodium and potassium concentrations across a cell membrane are of great importance in the maintenance of cellular activity. Moreover, potassium is implicated in the maintenance of the resting potential and an eleva-

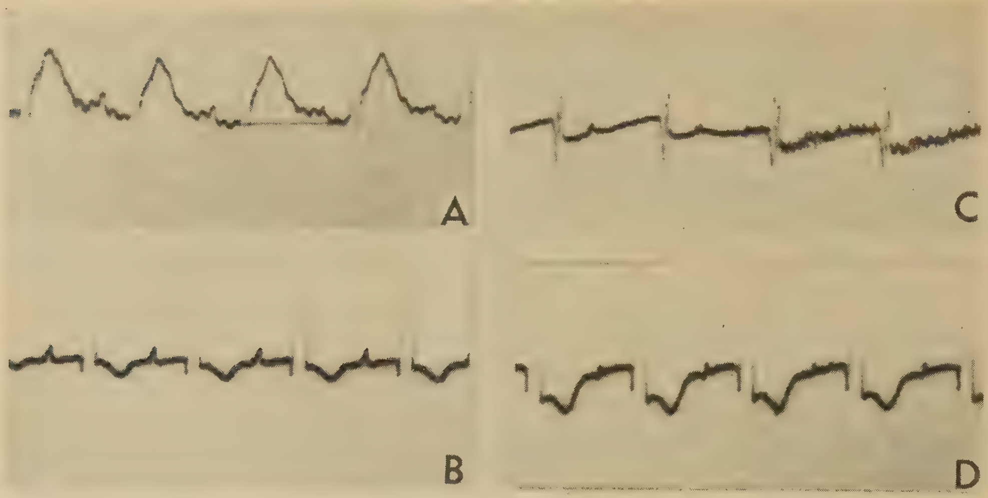

FIG. 1. ECG changes showing reversal of the T wave after adrenalectomy using lead VC-5 (A and B) and VL (C and D). A and C—before adrenalectomy (Na 140 mEq/l; K 6.6 mEq/l). B and D—three days after adrenalectomy (Na 95 mEq/l; K 15+ mEq/l).

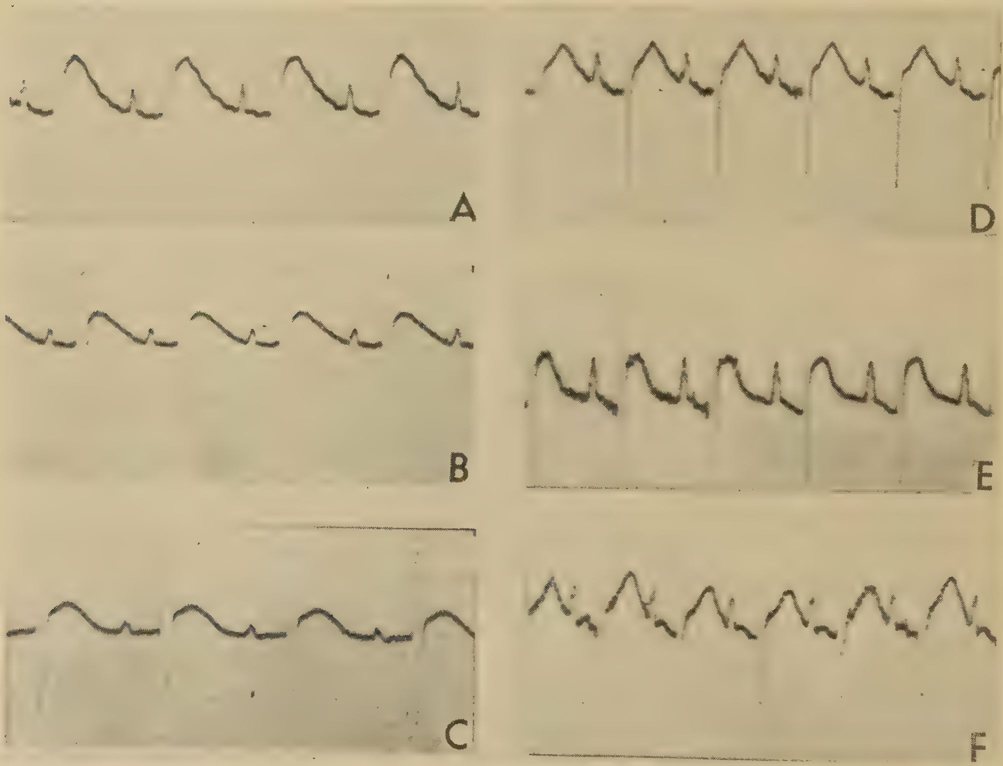

FIG. 2. ECG records (Lead VC-5) showing the effect of adrenalectomy (A, B, C) and cortisone-treatment (D, E, F) on normal hamsters. A. Before adrenalectomy (Na 155 mEq/l; K 5 mEq/l; heart rate 375/min.; T wave .33 mv; P wave .17 mv; QT 98 msec.). B. Two days after adrenalectomy (Na 141 mEq/l; K 10.5 mEq/l; heart rate 384/min.; T wave .24 mv; P wave .14 mv; QT 100 msec.). C. Five days after adrenalectomy (Na 123 mEq/l; K 12 mEq/l; heart rate 330/min.; T wave .2 mv; P wave .08 mv; QT 105 msec.). D. Before cortisone treatment. E and F. After 8 and 16 days of cortisone treatment; no significant changes.

TABLE II. Cortisone-Treated Hamsters; ECG, Serum Na and K.

Days of treatment	No. used and surviving	mEq/liter		Millivolts	
		Na	K	T wave	P wave
–3*	24	142 (130–150)	6 (4.2 –7.9)	.33 (.31–.35)	.12 (.11–.13)
3	23	150.2 (147–153)	5 (4.9 –5.3)	.28 (.27–.30)	.16 (.12–.20)
4	22	149 (141–151)	6.3 (4.7 –8.8)		
6	20	145.6 (137–152)	4.85 (4.25–5.5)	.28 (.20–.36)	.12 (.05–.21)
8	19	138.7 (122–152)	5.8 (4.1 –8.8)	.31 (.22–.40)	.21 (.14–.28)
10	15	146.2 (142–150)	5.8 (5 –8.8)	.32 (.27–.37)	.18 (.17–.19)
12	14	146.7 (145–150)	4.27 (3.8 –4.6)	.50 (.45–.52)	.22 (.16–.28)
14	8	134.5 (127–142)	5 (5)	.25 (.16–.37)	.17 (.11–.22)
16	6	139 (128–151)	6 (4.2 –7.7)	.45 (.37–.5)	.22 (.14–.28)
17	0				

* Three day pretreatment values for 24 hamsters in the group. During treatment, ECGs and heart punctures were made on 8 hamsters every other day.

tion of this ion or of lithium in the extracellular environment results in a depolarization of the cell(7,8).

Since vasoconstriction resulting from cortisone administration was not accompanied by significant alterations in either electrolyte levels or electrocardiograms, hyperpotassemia is not implicated as the cause. Our negative findings in the hamster differ from those of Overman who reported an increased plasma potassium in cortisone-treated dogs(9). After adrenalectomy, we found significant electrolyte and corresponding electrocardiographic changes in the absence of vasoconstriction. However, this does not obviate the possible role of hyperpotassemia during the terminal phase of adrenalectomy when compensatory vasoconstriction may be present. Important in this respect is the report by Katz and Lindner(3) that increased potassium concentrations in the dog produce sustained coronary constriction almost to the point of complete occlusion. However, it has been reported that a decrease in the plasma pH, bicarbonate ion, and sodium concentration will produce electrocardiographic alterations similar to those found during increased potassium concentrations (10).

Summary. Adrenalectomized hamsters exhibit an increase in the concentration of serum potassium and a decrease in serum sodium. These alterations in electrolyte levels are accompanied by changes in the electrocardiogram. The most significant modifications are bradycardia, a depressed T wave which in severe hyperpotassemia may be completely reversed, and an increased QT interval. Vasoconstriction in the cheek pouch does not necessarily accompany the hyperpotassemia of adrenal insufficiency. Moreover, cortisone-treated hamsters exhibited definite vasoconstriction, although there were no significant changes in the serum electrolyte levels or electrocardiograms.

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Received July 9, 1953. P.S.E.B.M., 1953, v84.

Angiotonin Induction of Vascular Lesions in Desoxycorticosterone-Treated Rats.* (20618)

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Injection of renin in rats pretreated with desoxycorticosterone acetate (DCA)(1,2), cortisone(3), or hydrocortisone(4) elicits an eclampsia-like state associated with acute vascular lesions. Similar lesions are also elicited by administration of renin to bilaterally nephrectomized dogs maintained on saline(5). However, there was no conclusive evidence that the renal pressor system was integrally concerned in eliciting these lesions. The renin preparations used were relatively impure, and furthermore, orienting experiments with small amounts of very crude angiotonin then at our disposal were unsuccessful in provoking the eclampsia-like syndrome and vascular lesions in rats pretreated with DCA(1).

The present brief report shows that angiotonin can elicit fluid retention and acute vascular lesions in rats pretreated with DCA and NaCl which resemble those precipitated by renin; from this we conclude that the metabolic and morphologic changes induced by renin in sensitized animals can justifiably be ascribed to the action of renin as such.

Methods. Female rats of about 150 g body weight were uninephrectomized; 2 35-mg pellets of DCA were implants subcutaneously and 1% NaCl was given as drinking fluid. On the 20th day, they were divided into 3 groups. The schedules of treatments follow: Group I (7 rats) received hourly intraperitoneal injections of angiotonin over the course of 12 hours; Group II (6 rats) received 3 subcutaneous injections of 0.5 cc of a renin preparation over 24 hours; Group III (5 rats) was given 0.9% NaCl intraperitoneally, hourly, for 12 hours. The total volume administered (4.8 cc) corresponded to that of the angiotonin solution given Group I. The animals were placed in metabolism cages immediately after the first injections. The functions observed were fluid intake and urine output, proteinuria (Shevky-Stafford method), and rectal temperature. The animals were sacrificed at the end of 24 hours, autopsied and tissues selected for histologic examination; the fixative was Zenker's, and the stains used were hematoxylin-eosin-methylene blue (H and E), periodic acid-fuchsin (PAS), and phosphotungstic acid hematoxylin (PTH). The renin preparation contained 15 mg protein per ml and its pressor activity was 1.9 Goldblatt dog units per mg. The angiotonin preparation had a pressor activity of about 70 dog units per ml; it was some 30 times more potent than the material used in the preliminary experiments previously described (1); estimates by ultraviolet spectroscopy indicated that it was about 10% as pure as Edman's best samples(6). Both the renin and angiotonin preparations were furnished by Dr. Arda A. Green.

Results. The data are summarized in

TABLE I. Functional and Morphological Effects of Angiotonin and Renin in DCA-Treated Rats.

Group and treatment	Body wt changes in g after 12 hr	24 hr	Fluid intake minus fluid output, cc	Urinary protein, mg/24 hr	Rectal temp.	Vascular lesions Incidence (%)	Severity*	Hemorrhages, incidence (%)
I Angiotonin	+35	+33	33	151	96	57	1.2	85
II Renin	+38	+52	72	185	87	100	2.6	100
III	— 5	+ 7	24	32	96	20	.2	0

* Graded from 0 to 4+.

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* This investigation was supported by a grant from the National Heart Institutes, National Institutes of Health, Bethesda, Md.

† Post-doctorate Fellow, Public Health Service.

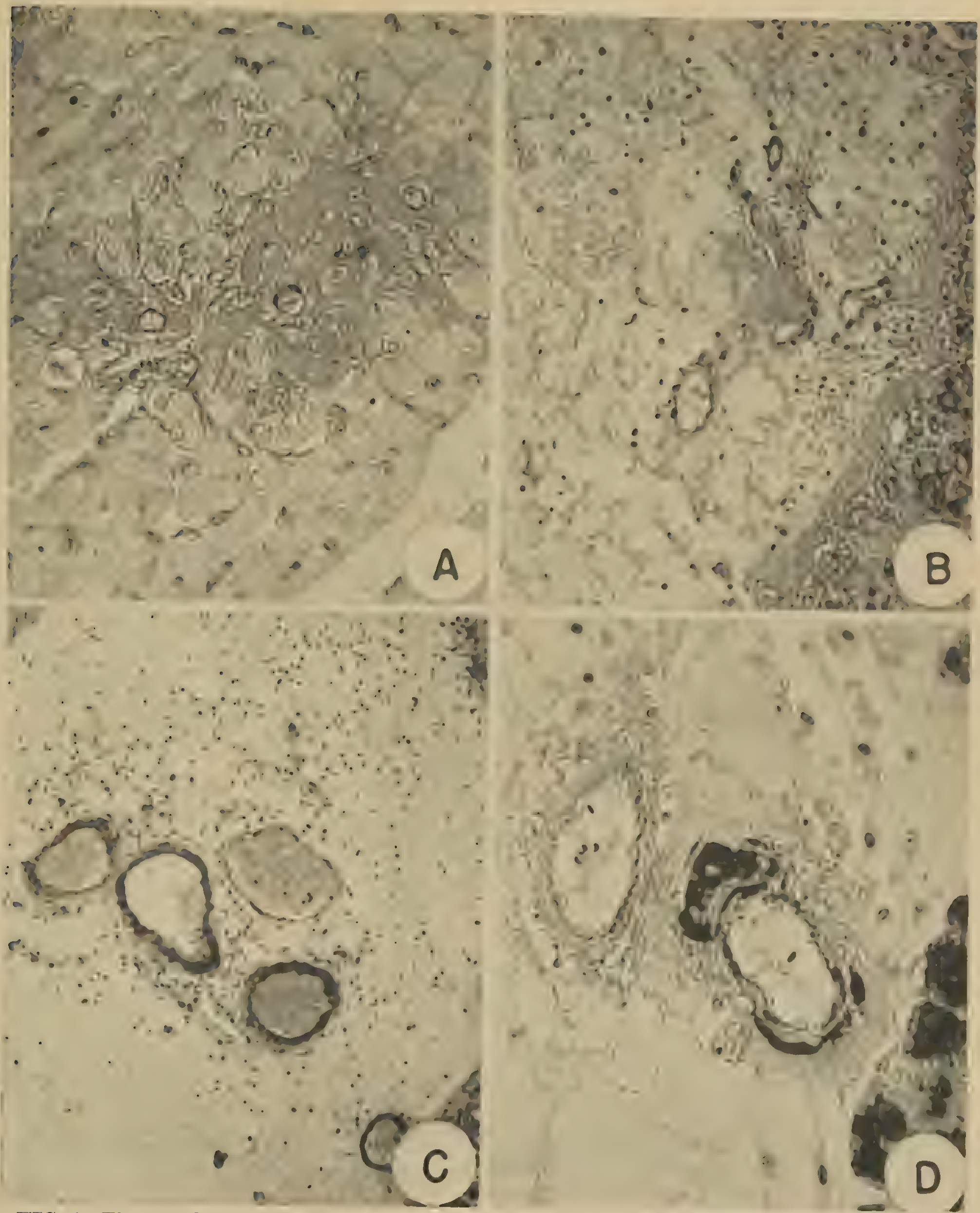

FIG. 1. Tissues of rats treated with DCA and angiotonin. (A) myocardium showing dilatation of small vessels and perivascular hyaline deposits which infiltrate between degenerating muscle fibers (×400, PAS); (B) stomach wall, showing edema, extravasation of erythrocytes and formation of hyaline deposit around a small vessel, portions of which seem dilated and necrotic (×200, PAS); (C) duodenal submucosa showing dilatation of hyalinized arterioles with stasis and edema (×120, PAS); (D) pancreatic arteriole with dilatation, perivascular exudate and intramural fibrinoid deposits (×400, PTH).

Table I. Animals of both Groups I and II demonstrated weight gain, large excesses of fluid intake over output, and proteinuria; rectal temperature decreased in the animals given renin. The prostration caused by the crude angiotonin formerly used was entirely absent in animals treated with the purer preparation.

The visceral hemorrhages characteristic of the gross appearance of the DCA-renin disease were found at autopsy in 6 of the 7 rats of the angiotonin group and in all the rats given renin. Two of the angiotonin-treated rats had convulsions; cerebral hemorrhages, to which these might be attributed, were not demonstrated at autopsy.

In the angiotonin group, 5 animals showed only subcutaneous edema; the other 2 showed also anasarca with fluid accumulations in serous cavities. Among the animals given renin, one failed to show edema, while the remaining 5 were in frank anasarca. Neither hemorrhages nor edema were demonstrable in the controls (Group III).

The severe renal vascular lesions previously described in DCA-renin disease were not demonstrable in animals of either Groups I and II. However, both groups did show hyalinization of large arterioles and necrosis of the wall of small arterioles with the smudged perivascular appearance of a PAS-positive "transudate". The myocardial and intestinal small arterioles showed the dilatation, fibrinoid necrosis, perivascular deposits and the associated necrosis of myocardial and smooth muscle tissues previously described. The relative severity of these changes was graded from one to 4+ and averaged for purposes of summary in Table I. Only minor glomerular, tubular and vascular lesions were found in animals of the control group.

Discussion. The data show that angiotonin can duplicate the essential phenomena of DCA-renin disease when a preparation of reasonable purity is administered in large doses at frequent intervals to rats pretreated with DCA and salt. The DCA-renin disease is widely variable in its severity between individuals; thus, none of the rats in this series developed its most severe and rapidly lethal form with anuria and thrombosis or necrosis of glomerular capillaries. This defect is perhaps fortunate for this demonstration, since the lesions caused by angiotonin and renin were more recognizably similar.

We would have preferred to elicit the condition in its severe form by administration of angiotonin. Unfortunately, there is no way of equating dosages of renin and angiotonin and it is very difficult to administer angiotonin in such a way as to simulate all the effects of the angiotonin liberated after subcutaneous injection of renin. The pressor effect could be reproduced by intravenous infusion of angiotonin; for this, the animal would require restraint and possibly anesthesia, both of which would interfere with the gratification of the thirst elicited by renin and the excess intake of saline which is one of the characteristics of the syndrome.

These experiments add to the similarities which have been shown to exist between the properties of renin and angiotonin; these include their pressor effects and their effects on fluid balance and proteinuria(7) and on electrolyte excretion(8). From the aspect of pathogenesis of the DCA-renin syndrome, salt intake and steroid treatment condition the production of vascular lesions by renin; this suggests that the lesions are somehow related to the effects of angiotonin on electrolyte distribution; the smudged perivascular deposits around small vessels seen in the DCA-renin and DCA-angiotonin treated rats seem to reflect an increased vascular permeability which is presumably the same as that which underlies the proteinuria induced by renal pressor agents.

Summary. 1. Hourly intraperitoneal administration of angiotonin to rats pretreated with DCA and salt elicited edema, convulsions, a positive fluid balance and increased fluid intake, together with proteinuria, visceral hemorrhages and acute necrotizing lesions in small arteries and arterioles. 2. The same series of changes occurred in similarly pretreated rats given renin subcutaneously. 3. We conclude that the vascular lesions elicited by renin in sensitized animals are very likely due to the action of renin as such, effected by liberation of angiotonin and are not, as hitherto seemed possible, attributable to vasculotoxic factors in renin preparations or to an action of renin independent of formation of angiotonin.

The desoxycorticosterone acetate was kindly furnished by Dr. R. Gaunt, Ciba Pharmaceutical Products, Inc., Summit, N. J.

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Received July 16, 1953. P.S.E.B.M., 1953, v84.

A Closed-Vacuum System for Harvesting Infectious Infant Mouse-Brain Material. (20619)

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The method developed by Casals, Olitsky, and Anslow(1) for obtaining high-titer complement-fixation antigen from infected suckling mouse brain has demonstrated the advantages of fewer anti-complementary and anti-mouse brain reactions in the serological study of certain neurotropic viruses. The method of antigen production, using a benzene extraction, has been adapted in this laboratory for the serological screening of human and vertebrate animal blood collected in Egypt and the Sudan (Taylor(2)) for immunity to West Nile and Ntaya viruses (Smithburn *et al.* (3); Smithburn and Haddow(4)).

The procedure, however, requires the tedious, time-consuming, and exposed harvesting of infectious mouse-brain material by laboratory personnel in order to obtain a sufficient quantity of material for extraction. These disadvantages have been partially overcome by a closed-vacuum, infant mouse-brain harvester developed in this laboratory.

Apparatus. The apparatus consists of a bluntly beveled 15-gauge needle attached by rubber tubing to a specimen vial and vacuum system. The needle is inserted into one end of a 6-inch section of catheter tubing; the other end of the tubing is attached to a right-angle glass tube that passes through one hole of a 2-hole rubber stopper placed in a specimen vial. The other hole of the stopper is connected to a vacuum system with an intervening trap flask containing chlorine solution. The needle and the rubber attachments are sterilized separately, and are connected with the specimen vial and vacuum system immediately before use. To assure instantaneous chilling and freezing of the brain material, the specimen vial is immersed in a bath of alcohol and CO_2 ice.

Technic. The mice whose brains are to be harvested are placed under deep ether or chloroform anesthesia and, if desired, exsanguinated. The head is cleansed with alcohol and the needle passed through the occiput of the skull. The vacuum, which is controlled by pressing the catheter tubing over the end of the needle attached to the tubing, is then applied by releasing the pressure, and the brain matter is sucked through the needle and tubing and deposited in the specimen vial. The aspiration of the brain is evidenced by the collapse of the skull. The vacuum is then closed until the next brain is harvested. A hundred mouse brains can be harvested completely in less than an hour by this method. Not only does it serve quickly and efficiently to collect all brain material, but it prevents open exposure of virulent material to persons harvesting highly infectious virus. The above technic has been used successfully in this laboratory to harvest brain material from infant mice up to 12 days of age.

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Summary. A safe, time-saving, closed-vacuum harvesting technic is described. The advantages and safeguards of this technic are enumerated.

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Received August 5, 1953. P.S.E B.M., 1953, v84.

Improved Method for Determination of Plasma Polysaccharides with Tryptophan.* (20620)

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(Introduced by Morris Ziff.)

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There is currently an active interest in the determination of the polysaccharide content of plasma and other biological fluids as related to different diseases. The tryptophan reaction proposed by Shetlar, Foster, and Everett(1) has, for clinicians, certain advantages over the orcinol reaction of Sorensen and Haugard(2). The stock tryptophan solution can be kept for several days on ice; the production of the color at 100°C does not call for the greater care required in the orcinol reaction which must be run at 80°C; and the color produced is less affected by impurities in the reagents. As described, the tryptophan reaction has the serious failing of giving colored products whose absorption spectra differ widely with different sugars. For instance, glucose gives a yellowish-brown color while mannose gives a pure violet. It will be shown that when the reaction is carried out in the presence of boric acid this failing is diminished. The best results are obtained with the highest possible concentration of boric acid.

Reagents. 1) Absolute ethanol. 2) Boro-sulfuric acid, containing sulfuric acid (770 ml), water (230 ml) and boric acid (50 g). For comparison, a sulfuric acid solution is prepared omitting the boric acid. 3) Tryptophan (1 g) dissolved in boiling water (100 ml). The L-form is preferable to the DL-form because it is more soluble and solutions kept on ice do not crystallize. 4) Standard mannose solution (50 to 150 μg/ml). This can be kept on ice for several weeks in the presence of a few drops of toluene.

Procedure. The reaction is best carried out in carefully cleaned matched tubes that are to be used in the photometer. Serum (.05 ml) is introduced, and then absolute alcohol (about 10 ml). After 15 minutes the tube is centrifuged at 2500 rpm for 10 minutes, the alcohol is decanted, water (1 ml) is added and the precipitate is dispersed. From a burette, boro-sulfuric acid solution (7 ml) is added and the contents of the tube are immediately mixed and chilled in ice water for 5 minutes. After leaving the tube at room temperature for 15 minutes, the tryptophan solution (1 ml) is added. The contents of the tube must be mixed immediately. Because of the high density of the sulfuric acid solution this is best done by bubbling air through it. The tube is again chilled in ice water. At the same time a series of 6 or 8 tubes is similarly prepared containing different amounts of the standard mannose solution, each made up to 1 ml before addition of the boro-sulfuric acid. All the tubes are heated in a boiling water bath for 20 minutes, and the contents again mixed once or twice during this time. The tubes are then

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* This work was supported by research grant from the National Institute of Arthritis and Metabolic Diseases, of the National Institutes of Health, Public Health Service, and by the Masonic Foundation for Medical Research and Human Welfare.

† Aided by a Fulbright Smith-Mundt Fellowship.

TABLE I. Comparison of Wave Lengths of Maximum Absorption and Optical Densities, at These Wave Lengths, Produced by Different Sugars in the Sulfuric Acid and the Boro-sulfuric Tryptophan Reactions.

Sugar	Wave length (mμ) of max. absorption: Sulfuric acid	Wave length (mμ) of max. absorption: Boro-sulfuric acid	Optical density/100 μg sugar at max.: Sulfuric acid	Optical density/100 μg sugar at max.: Boro-sulfuric acid
Glucuronic acid	480	540	.258	.393
Glucose	440	525	.504	.703
Arabinose	480	520	.526	.680
Galactose	480	520	.478	.586
Mannose	500	520	.586	.700
Fucose	500	520	.588	.646

chilled for 5 minutes in a cold water bath and left at room temperature for a half hour. Optical density is measured at 520 mμ or with a green filter. Some brands of sulfuric acid gave a marked yellow color when heated with the tryptophan solution. Only sulfuric acid that gives no more than a faint yellow tint should be used. The tryptophan solution, stored on ice, should not be kept for more than 10 days. If the tube contents are not mixed immediately after the addition of the tryptophan, a false yellow color develops in the zone where tryptophan and boro-sulfuric acid meet. In all work described, the optical density is corrected by subtraction of the optical density of an identically made solution containing no sugar. An advantage of the boro-sulfuric acid over the sulfuric acid reagent lies in the fact that this correction is smaller. In a 1 cm cell this correction is .192 at 425 mμ, .092 at 475 mμ, and .052 at 525 mμ.

Results. Six sugars were examined by the method described. In all cases the color produced in boro-sulfuric acid is a pure violet compared to the more variable brown to violet colors produced in sulfuric acid. For each sugar the plot of corrected optical density against the amount of sugar used is linear up to 150 μg. This is true in the absence as well as in the presence of boric acid. The data are not presented in detail but the slope of each of these lines is given in Table I as well as the wave length at which maximum optical density occurs. The spectra of the colors produced by each of the sugars in the absence and presence of boric acid are shown in Fig. 1. In this reaction glucosamine and galactosamine produce no color at all.

Since the main purpose of this work was to provide a reliable and quick assay method for the polysaccharides in plasma it became necessary to ascertain whether the presence of boric acid would interfere with the hydrolysis of these polysaccharides or the rate of color development. That boric acid does not interfere with either process is shown in the curves of Fig. 2.

The thought of Sheppard and Everett(3) underlying the development of the reaction of sugars with tryptophan seems to have been that the color was due to the formation of black humin from furfural and tryptophan. This idea was tested by observing the color developed with furfural and tryptophan in the absence and in the presence of boric acid. For this purpose furfural was freshly distilled and a solution made containing 100 μg per ml. Its behavior was the same in the absence or presence of boric acid; a bright yellow color is produced instantly in the cold on the addition of tryptophan, and this is converted after 20 minutes of heating at 100°C to a reddish-brown color. No trace of violet color is produced as occurs with all the sugars tried. It must be concluded that the violet color is not a result of furfural formation from any of the sugars. This was true even of glucuronic acid which does yield furfural on heating with sulfuric acid. If glucuronic acid is first heated with either the sulfuric or boro-sulfuric solution used in the test and then cooled, addition of tryptophan produces the same yellow color as with furfural and only a brown color develops on heating at 100°C. Experiments

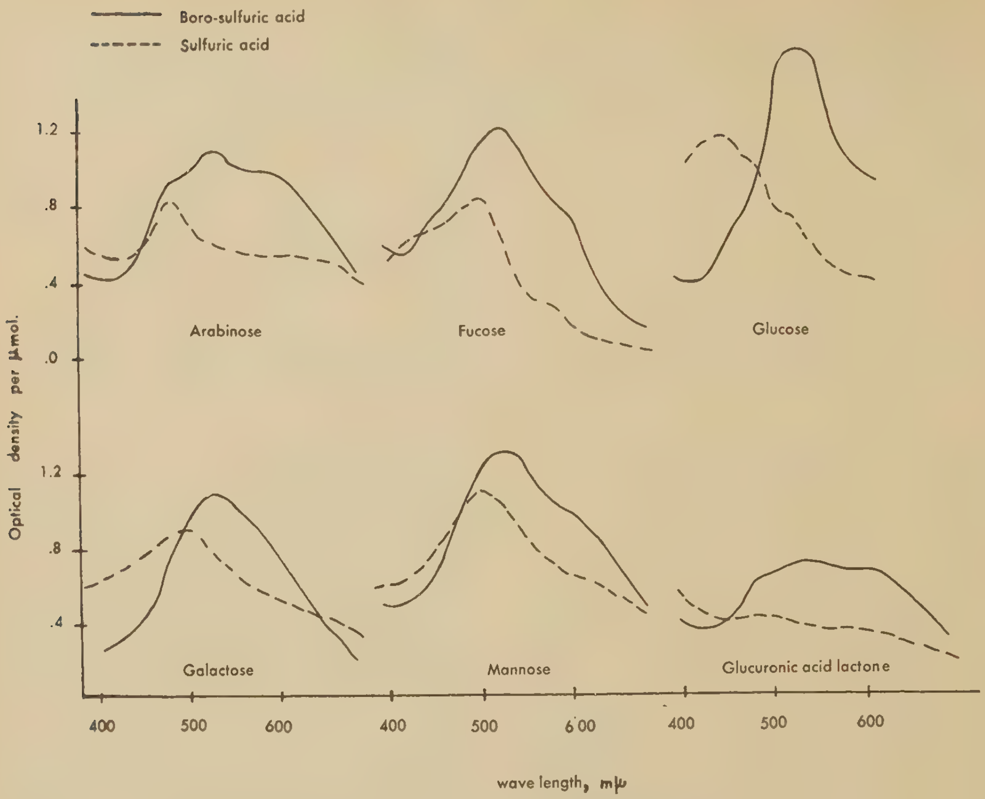

FIG. 1. Comparison of spectra of colored products resulting from tryptophan reaction in sulfuric acid solution and in boro-sulfuric acid solution.

were also carried out using a 1% solution of indole in 50% ethanol instead of the tryptophan solution. Only brown colors were produced which in a few cases were intensified by the presence of boric acid.

It has also been found that if the colors are developed by allowing the reaction mixtures to stand for 24 hours at 37°C instead of heating for 20 minutes at 100°C, then all the sugars develop violet colors with maxima at 520 to 540 mµ even in the absence of boric acid. Under these conditions the presence of boric acid also produces a higher color density.

Discussion. The advantages of this new tryptophan method are that there is an increase in optical density for each sugar, that the maximum optical density occurs at longer wave lengths where the blank correction is smaller, that the wave lengths of optical density are more nearly the same for different sugars, and that there is a small decrease in the difference in color density produced by different sugars.

The striking effect of boric acid on the color produced by the reaction of tryptophan with sugars might be associated with the ability of boric acid to form complexes with sugars and to protect them from side reactions with the sulfuric acid. But in acid medium boric acid seems not to form complexes with sugars according to work of Rumbach and Weber(4), Boeseken(5), and Levy and Doisy(6). In alkaline solution Levy and Doisy(7) found that glucose was protected against oxidation by alkaline hypoiodite to a greater extent than arabinose and galactose. This is the same as the order found in the present work for the extent of the effect of boric acid on the color produced in the tryptophan reaction.

Because of the absence of evidence for a reaction between boric acid and sugar in the acid medium, another thought was considered to account for the improvement in color it produced. Boric acid reacts with sulfuric acid

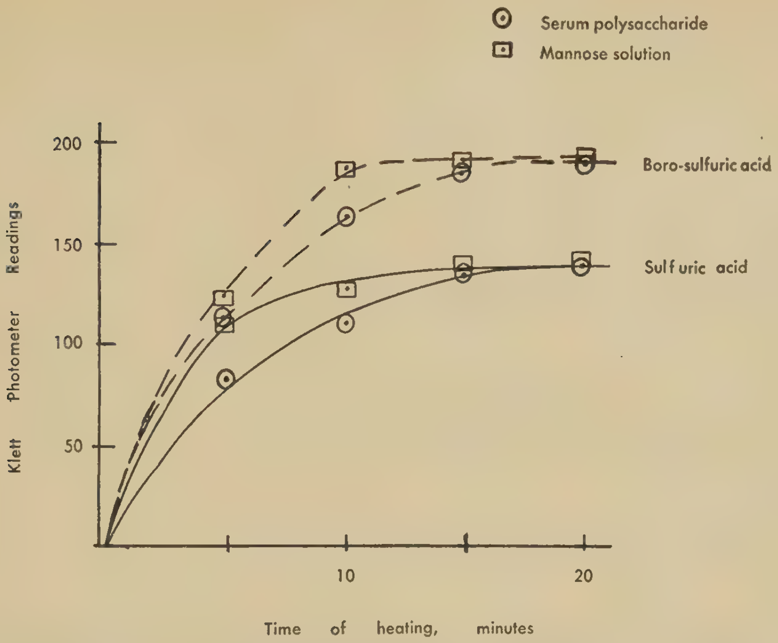

FIG. 2. Comparison of rate of color development in a serum sample and in a standard mannose solution when the tryptophan reaction is run in sulfuric acid solution and in boro-sulfuric acid solution. Protein from .05 ml normal serum was precipitated with ethanol (10 ml) and the tryptophan reaction was carried out on this precipitate. For comparison, the reaction was also carried out on 45 μg of mannose which yields the same final optical density. The color develops slightly more slowly in the serum due to progressive liberation of sugars, but this is not influenced by boric acid.

to produce borylsulfates. This raised the question whether borylphosphates or borylacetates might not also bring about such an improvement. Trial of a variety of mixtures of phosphoric or acetic acids with sulfuric acid and with or without boric acid, however, yielded no improvement in color.

Summary. A method to measure the concentration of sugars in solution, particularly of polysaccharides in biological fluids such as plasma, is presented. It is a modification of a tryptophan method already described and utilizes boric acid in the reaction medium. The color intensity is increased and the color quality modified to give maximum absorption at a longer wave length where interference by non-specific brown produced by reagent blanks and proteins is diminished.

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Received August 6, 1953. P.S.E.B.M., 1953, v84.

Relationship of Pyridoxine and its Derivatives to the Mechanism of Action of Isoniazid.* (20621)

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In most instances of metabolic antagonism the essential metabolite and the inhibitory agent have been similar in chemical structure. The structural appearance of isoniazid‡ lead to the speculation that its mode of action might represent interference with the utilization of nicotinamide or some other essential metabolite of similar structure. This report deals with the screening of certain vitamins, some of which are structurally similar to isoniazid, for possible isoniazid reversal.

Materials and methods. The method chosen to study the mode of action of isoniazid was similar to that of McIlwain(1), and Lampen and Jones(2). Various organisms with known nutritional requirements were inhibited with the drug and reversal of the inhibition was initially attempted with nicotinic acid, nicotinamide, pyridoxine, thiamine, riboflavin, pantothenic acid, and p-aminobenzoic acid. Studies were later limited to pyridoxine and its derivatives when the initial results showed that only these compounds gave a competitive reversal of inhibition. The final steps were to study the effects on various species of *Mycobacterium*. No attempt was made to study any organism in any medium when a concentration of greater than 10^{-2} M of the isoniazid was required for inhibition. The organisms employed in this study were obtained from the American Type Culture Collection, Washington, D.C., unless otherwise stated. Synthetic media were used whenever possible to control the amount of essential metabolites present. Some comparisons were made between synthetic and nonsynthetic media and Table I lists the organisms, media, references, and the method of measuring and detecting growth. *Lactobacillus plantarum* (*arabinosus*) 17-5 was chosen because of its wide external nutritional requirements which included those of major interest, nicotinic acid and pyridoxine. It also discriminates between pyridoxine and its derivatives, pyridoxal and pyridoxamine, the latter 2 being more active than pyridoxine as growth promoters. In all experiments using *Lactobacillus* changes in growth were observed grossly over 72 hours and the medium titrated for acid production at that time. *Streptococcus pyogenes* was used only because of its external pyridoxine requirement. *Saccharomyces carlsbergensis* was chosen because of its use in assay of vit. B_6 and its uniform response to B_6 and its derivatives. Final turbidity readings for determination of growth were made after 18 hours incubation at 30°C. The effect of isoniazid inhibition was studied in relation to the growth of 2 *Escherichia coli* strains which do not require added B_6, one mutant strain of which requires nicotinic acid or its amide. The final turbidity readings were made after 18 hours incubation at 37°C. The *E. coli* strains are similar to the *Mycobacterium* which also have no external requirement for pyridoxine or its derivatives. Both are known to synthesize vit. B_6. *Mycobacterium butyricum* grows rapidly in a synthetic media and has been used for the assay of isoniazid(6). Cultures were observed for growth at the end of 24 and 48 hours and the end of one week. The BCG and H37Rv strains were incubated for at least 3 weeks before final growth and turbidity observations were made.

Results. Using the standard technic of microbiological assay with *Lactobacillus plantarum* and its synthetic medium containing all the maximum growth requirements, it was possible to inhibit the organism with isoniazid. Inhibition began with isoniazid concentrations of 4.8 x 10^{-3} and was complete at 10^{-2} M. Table II gives the concentrations of the isoniazid in the medium with the acid titration values produced after 72 hours growth.

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* Work done under the auspices of the AEC.

† Sandia Base with station at Los Alamos.

‡ Kindly supplied as Nydrazid by Squibb and Co., and as Rimifon by Hoffmann-LaRoche, Inc.

TABLE I. List of Microorganisms, Media, and Method of Measuring Growth.

Microorganism	Growth medium	Method of measuring growth response
Lactobacillus plantarum (arabinosus) 17-5 ATCC No. 8014	Modified Snell and Wright synthetic(3)	Titration of acid produced*
Streptococcus pyogenes ATCC No. 10389	Meat infusion broth	Gross growth observations
Escherichia coli†	Nutrient and tryptone broths	Turbidity‡
Escherichia coli strain 303-138 nicotinic acid mutant ATCC No. 9723†	Synthetic medium of MacLeod plus nicotinic acid(4)	"
Saccharomyces carlsbergensis ATCC No. 9080	Synthetic medium as used in vit. B_6 assay(3)	"
Mycobacterium tuberculosis var. bovis BCG§	Modified Kirchner's synthetic (5) and Dubos medium	Gross growth observations and turbidity
Idem., var. hominis H37Rv ATCC No. 9360	Dubos medium	*Idem.*
Mycobacterium butyricum ATCC No. 362	Synthetic as used in assay of isoniazid(6)	Gross growth observations pellicle type

* All titrations were done with 0.1 N NaOH.
† Isolated in this laboratory.
‡ Turbidity readings on Coleman Model 14 Universal Spectrophotometer.
§ BCG strain obtained from Chicago Research Laboratories.

TABLE II. Concentrations of Isoniazid Used to Obtain Inhibition of *Lactobacillus plantarum.*

Conc. of isoniazid, M	ml of 0.1N NaOH/10 ml*
0	1.8†
0	12–13‡
7.3×10^{-4}	12.5
3.65×10^{-3}	5.0–6.5
5.80×10^{-3}	3.0–3.8
7.30×10^{-3}	2.5–3.3
1.0×10^{-2}	1.2–2.8

* Avg range of titration results at 72 hr, all determinations.
† Control in which basal medium was deficient in nicotinic acid.
‡ Control, complete medium with all essential growth requirements present.

Table III is a summary of the results obtained when reversal of the isoniazid inhibition in *Lactobacillus* was attempted with some of the more common metabolites. The only substance tested that had any effect was pyridoxine. Inhibition by a concentration of 10^{-2} M of isoniazid was completely reversed by 4.8 x 10^{-4} M of pyridoxine when added to the complete synthetic medium.

Table IV demonstrates the reversal of the isoniazid inhibition in *Lactobacillus* with varying concentrations of pyridoxine and its derivatives. A competitive type of reversal is demonstrated. The data show that the derivatives of pyridoxine, pyridoxamine, and pyridoxal, were much more effective in reversing the isoniazid inhibition. Pyridoxamine dihydrochloride was from 2000-4000 times as effective as pyridoxine while the pyridoxal hydrochloride was 1000-2000 times as effective in reversing the inhibition of 10^{-2} M concentration of isoniazid.

With *Saccharomyces carlsbergensis* it was possible to demonstrate again the competitive reversal of the isoniazid by pyridoxine and

TABLE III. Common Metabolites Tested for Reversal of Isoniazid Inhibition of *Lactobacillus plantarum.*

Conc. of isoniazid, M	Metabolite	Added conc., M	Growth*
0	Complete basal medium†		4+
1×10^{-2}	" " "		—
"	Nicotinic acid	$.04–4.0 \times 10^{-3}$	—
"	Nicotinamide	$.04–4.0 \times$ "	—
"	Pyridoxine·HCl	$.24–4.8 \times 10^{-4}$	3+–4+
"	Thiamine·HCl	$.15–3.0 \times$ "	—
"	Ca pantothenate	$.11–2.3 \times$ "	—
"	p-aminobenzoic acid	$.37–3.7 \times$ "	—
"	Riboflavin	$.13–1.3 \times$ "	—

* Determined by titration of acid produced.
† All essential metabolites present in basal medium in conc. of no more than 2 μg/10 ml.

TABLE IV. Competitive Reversal of Isoniazid Inhibition of *Lactobacillus plantarum* by Pyridoxine and Its Derivatives.

Conc. of isoniazid, M	Metabolite conc., M	ml of 0.1 N NaOH/10 ml of media
	Pyridoxine • HCl	
0	9.7×10^{-7}	12.7
1×10^{-2}	9.7×10^{-7}	2.3
"	4.8×10^{-6}	2.6
"	9.7×10^{-6}	2.8
"	2.4×10^{-5}	3.1
"	4.8×10^{-5}	3.4
"	2.4×10^{-4}	10.7
"	4.8×10^{-4}	11.4
"	1.9×10^{-3}	12.6
	Pyridoxamine • 2HCl	
0	*	13.1
1×10^{-2}	*	1.1
"	4.1×10^{-9}	4.6
"	2.1×10^{-8}	8.3
"	4.1×10^{-8}	10.0
"	1.2×10^{-7}	11.6
"	2.1×10^{-7}	12.8
"	4.1×10^{-7}	13.8
"	8.2×10^{-7}	13.8
	Pyridoxal • HCl	
0	*	13.3
1×10^{-2}	*	2.8
"	4.9×10^{-8}	4.3
"	1.5×10^{-7}	5.1
"	2.5×10^{-7}	7.2
"	4.9×10^{-7}	7.9
"	9.8×10^{-7}	8.5
"	2.5×10^{-6}	11.8
"	4.9×10^{-6}	12.8

* All basal media contained 2 μg (9.7×10^{-7} M) pyridoxine/10 ml.

its derivatives. In this organism, however, no great differences were noted between pyridoxine, pyridoxamine, and pyridoxal, except that the 2 latter derivatives were slightly less effective. Table V demonstrates some of the results when the concentration of metabolite was constant and the amount of inhibitor varied. Similar competitive reversal of inhibition was obtained when the inhibitor concentration was constant and the metabolite varied.

The first 2 organisms discussed above are representative of the type that requires an external source of pyridoxine. Table VI shows some results with the laboratory strain of *Escherichia coli* which is an example of the type without external pyridoxine requirement. The strain isolated in the laboratory was inhibited in nutrient broth by the amounts of isoniazid shown in the table and reversal by pyridoxine and its derivatives was only partial. Pyridoxal and pyridoxamine appeared only slightly more active in reversing the inhibition than did pyridoxine.

Results with the mutant strain of the *E. coli* requiring nicotinic acid were slightly different. Pyridoxine at no time reversed completely the inhibition. Pyridoxamine and pyridoxal produced a complete reversal of inhibition and were at least 2 to 3 times more active.

It is interesting to observe that *Streptococcus pyogenes* could not be inhibited by isoniazid in any concentration up to 10^{-2} M. This was attempted in the commonly used meat infusion broth. Although this organism

TABLE V. Competitive Reversal of Isoniazid Inhibition by Pyridoxine • HCl in *Saccharomyces carlsbergensis*.

Conc. of pyridoxine, M	Conc. of isoniazid, M	Opt. density at 650 mu
0	0	.1
4.86×10^{-5}	0	1.88
"	7.3×10^{-4}	1.80
"	2.19×10^{-3}	1.75
"	$2.9 \times$ "	1.45
"	$3.65 \times$ "	1.24
"	$5.8 \times$ "	.79
"	$7.3 \times$ "	.65
"	1.0×10^{-2}	.32

TABLE VI. Inhibition of *E. coli* (Laboratory Strain) with Isoniazid and Partial Reversal of Inhibition with Pyridoxine and Pyridoxamine.

Conc. of isoniazid, M	Conc. of metabolite, M	Optical density†
	Pyridoxine • HCl	
0	0	.57
5.8×10^{-3}	0	.12
"	4.8×10^{-5}	.19
"	2.4×10^{-4}	.19
"	$4.8 \times$ "	.22
"	1.44×10^{-3}	.28
"	$2.9 \times$ "	.36
	Pyridoxamine • 2HCl*	
0	0	.55
5.8×10^{-3}	0	.11
"	4.1×10^{-5}	.16
"	2.1×10^{-4}	.21
"	$4.1 \times$ "	.24
"	1.26×10^{-3}	.36
"	$1.7 \times$ "	.45

* Pyridoxal gave similar results up to concentrations of 2.45×10^{-4} M.

† All measurements made at 620 mu.

has an external requirement of pyridoxine, it was felt that in all probability the non-synthetic medium contained sufficient pyridoxine or its derivatives to prevent any inhibition. Inhibition of the common strain of *E. coli* was also attempted in tryptone broth without success. Tryptone broth may have sufficient vit. B_6 or one of its derivatives to compete with the isoniazid. Although nutrient broth is not a synthetic medium its vit. B_6 content may be sufficiently low to have little effect on the isoniazid inhibition. The initial amount of pyridoxine and its derivatives in many common laboratory media may account for the wide discrepancies in reports of inhibition of other microorganisms by isoniazid.

In an attempt to study the reversal of isoniazid inhibition in the various strains of *Mycobacteria* no effect of any nature was noted. Using the BCG strain in modified Kirchner's and Dubos medium and the H37Rv strain in Dubos medium, inhibition by concentration of isoniazid from 0.073 to 7.3×10^{-5} M was not reversed by concentrations up to 1×10^{-3} M of pyridoxine and its derivatives. In the *M. butyricum* using the synthetic isoniazid assay medium no reversal was noted when the concentrations of isoniazid ranged from 3.6 to 36×10^{-5} M and the concentration of pyridoxine and its derivatives ranged up to 5×10^{-4} M.

Discussion and conclusions. Of the more common vitamins tested for reversal of isoniazid inhibition in *Lactobacillus plantarum*, nicotinic acid, nicotinamide, and pyridoxine and its derivatives were structurally similar to isoniazid. The concentrations of isoniazid required to inhibit *Lactobacillus* and the other organisms studied were much greater than are required to inhibit *Mycobacteria*. Although relatively high concentrations of isoniazid were required to inhibit *Lactobacillus plantarum* and *Saccharomyces carlsbergensis*, when pyridoxine and its derivatives were supplied externally, a typical competitive type reversal of the inhibition was obtained. With *Lactobacillus*, pyridoxal and pyridoxamine were much more effective than pyridoxine in reversing this inhibition. This result may be expected as the lactic acid bacteria normally demonstrate a greater response to pyridoxamine and pyridoxal. With the strains of *E. coli*, larger amounts of vit. B_6 and derivatives were required to produce reversal of inhibition and reversal was not always complete and not as typically competitive. The fact that *E. coli* can synthesize pyridoxine may result in a more complex, atypical mode of inhibition and reversal. Using *Mycobacteria*, which synthesize pyridoxine, no reversal of isoniazid inhibition was obtained by addition of any amounts of pyridoxine or its derivatives to the media. If vit. B_6 is involved in the inhibition of growth of *Mycobacterium* by isoniazid it does not appear to be a substrate phenomenon as in the case of *Lactobacillus* and *Saccharomyces*. The above results, however, do not preclude the possibility of a metabolic antagonism between isoniazid and vit. B_6. It is possible that in the *Mycobacteria* isoniazid may produce inhibition by interfering with the initial synthesis of pyridoxine or by interfering with the utilization of pyridoxine or its derivatives for the synthesis of essential substances such as enzyme systems in which they serve as prosthetic groups, as in decarboxylase or transaminase reactions. Zeller and Barsky(7,8) demonstrated inhibition of monamine oxidase on bacterial and mammalian enzymes with isoniazid and isonicotinyl-2-isopropyl hydrazine. No attempt to reverse this inhibition was mentioned. Yoneda *et al.*(9,10) were able to reverse isoniazid inhibition of indole formation and amino acid decarboxylation activity in *E. coli* with vit. B_6 and its derivatives. The present results also indicate that isoniazid is an antimetabolite of vit. B_6 in certain microorganisms. Antagonism between isoniazid and vit. B_6 was not manifested in the *Mycobacterium* however, and does not serve to explain the mode of action of isoniazid on the tubercle bacillus.

Preliminary results in this laboratory(11) indicate that the uptake of C^{14} labeled isoniazid by these organisms is very small and as yet no conclusion can be drawn in relationship to the mode of action of isoniazid.

Summary. 1. It was possible to inhibit growth of organisms other than *Mycobacterium* with isoniazid by controlling the amount of pyridoxine and its derivatives in the media. *Lactobacillus plantarum* and *Saccharomyces carlsbergensis* were inhibited in a synthetic medium by isoniazid concentrations of less than 10^{-2} M. This inhibition was competitively reversed by pyridoxine, pyridoxamine, and pyridoxal. In reversing isoniazid inhibition of *Lactobacillus,* pyridoxamine, and pyridoxal were from 1000 to 4000 times more effective, than pyridoxine. 2. Two strains of *E. coli* were inhibited with isoniazid with partial reversal with pyridoxine and derivatives. Larger amounts of the metabolite were required and the reversal was not as typically competitive. 3. No reversal of isoniazid inhibition could be obtained with vit. B_6 or its derivatives in any of the *Mycobacteria* tested. If isoniazid acts as an antagonist of pyridoxine or its derivatives in the *Mycobacteria,* it is not a simple substrate competition or uptake phenomenon.

Acknowledgement is made with thanks to Mrs. Donna Faye Turney for her technical assistance.

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Received August 7, 1953. P.S.E.B.M., 1953, v84.

Failure of Grafted Ovaries to Assume Adrenalcortical Function in Rats.*
(20622)

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It has been shown that under specific conditions the ovary of the mouse can and does assume life-sustaining corticoadrenal functions(1,2). Working independently and in different laboratories, the authors have made autoplastic intrasplenic ovarian grafts in young adult and in immature rats in an effort to determine the effectiveness of such a graft on post adrenalectomy survival time in the rat.

The recent article by Lichton, Goldblatt, and Stolpe(3) prompts us to make our data available.

Methods. The animals used were of sturdy albino stock of no specific strain origin, and in each case were fed standard laboratory diets. The experiments on the young adult rats were performed by Hill, and those experiments on the immature rats were done by Bernstorf. The mature rats were bilaterally spayed, and at the same time the left ovary was grafted into the spleen. At various intervals following recovery, the right adrenal was removed. The left and second adrenal was removed approximately 2 weeks following first adrenal removal (Table I). Following the completion of adrenalectomy, the weights of all animals were recorded every few days. If the animals were discovered soon after death they were examined for remnants of adrenal tissue and for the general condition of the graft. The immature rats were spayed at weaning, and 17 of the 19 animals used

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* This project was supported, in part, by funds from the U. S. Public Health Service to R. T. Hill under grant.

TABLE I. Body Weight and Length of Post-adrenalectomy Survival in 22 Mature Graft-Bearing rats.

Body wt in g						
Day postadrenalectomy				Last wt		No. of survival days
1	6	13	15	Day	g	
177	170	146	149	20	115	20
194	174	170	165	20	144	20
—	—	—	—	—	—	1*
—	—	—	—	—	—	1*
178	172	160	149	—	—	20
215	203	202	200	34	186	34
159	157	140	141	24	131	28
157	143	127	122	16	116	16
167	152	161	157	24	134	28
—	—	—	—	—	—	1†
168	157	143	141	21	115	21
Day postadrenalectomy						
2	6	13	17			
182	169	167	—	—	—	16
178	170	175	172	24	140	24
153	144	137	126	21	115	21
203	185	180	169	48	157	48
203	193	190	173	19	150	19
161	150	137	124	21	109	21
168	162	150	137	23	123	23
192	179	176	164	24	144	28
193	190	210	206	24	208	24
163	151	147	130	23	90	23
154	140	142	129	19	114	23

* Operated on by technician, died 1st post op. day and excluded from results.

† Eviscerated through surgical opening.

were given an ovarian autograft into the spleen. Two animals were left without ovarian graft as controls. This entire group was adrenalectomized in 2 stages on days 30 and 34 following the grafting of the ovary into the spleen. All animals were weighed at the time of removal of the final adrenal, and again after 5 days. At death the animals were observed for adrenal tissue and the grafts of ovaries were weighed (Table II).

Data and discussion. In the adult rat series, 17 of 22 animals died within 30 days after adrenalectomy, with an average survival time of 22 days. Three animals died on the first post operative day (see footnote to Table I), and therefore are excluded. Two animals survived adrenal removal for periods of 34 and 48 days, respectively, and if included in the average survival time, they augment it upwards by only 2 days.

The immature animals had an average survival time of 10 days, with the ungrafted adrenalectomized controls having a survival of 7.5 days.

We note with interest that the average survival period of all of our ovarian grafted rats is quite in line with the average survival time of the adrenalectomized control animals of Lichton *et al.* Furthermore, all of our animals were observed to carry macroscopic grafts at the time of removal of the second adrenal (left), and in most of the immature animals the graft was weighed following death.

It is impossible to explain the differences in the results obtained by us and those obtained by Lichton and coworkers(3). For the present those differences must be ascribed to one or more unknown factors, which it is hoped will be cleared by subsequent studies. We believe it is significant that our results bear such remarkable correlation especially when the work was done in 2 different laboratories, with an intervening period of more than 2 years in the 2 sets of experiments, and by 2 separate investigators on 2 totally unrelated groups of rats. We take note of the effectiveness of ovaries in mice in prolonging life after adrenalectomy(1,2). Likewise we are well

TABLE II. Body and Ovarian Graft Weights and Length of Postadrenalectomy Survival in 19 Immature Graft-Bearing Rats.

Body wt in g			
Day postadrenalectomy		Graft wt in mg	No. of survival days
0	5		
111.8	106.4	8.7	7.0
114.3	118.7	13.4	12.5
104.6	115.7	31.2	12.5
119.8	122.2	77.8	10.0
117.0	110.4	72.5	22.5
118.4	120.9	4.3	12.0
116.0	117.3	14.9	12.0
102.4	108.8	69.2	13.5
123.2	105.8	39.4	7.5
109.5	109.1	28.4	10.5
102.0	122.5	—	—
104.5	105.4	22.7	11.0
102.9	99.7	54.2	7.0
97.7	98.8	67.1	7.5
88.9	83.6	64.8	8.5
94.4	85.0	66.3	8.5
110.0	96.8	97.6	8.0
Adrenalectomized controls			
101.5	86.2	—	7.5
90.0	88.5	—	7.5

aware of the androgenic potentialities of the mouse ovary(4,5).

Deanesly(6) and subsequent workers have extended Hill's original observations, finding an androgenic activity in the rat ovary. However, in no case has the rat exhibited the same quality of androgenic activity in its ovaries as that found in the mouse. Likewise the androgenic response in the mouse ovary is much more closely correlated with temperature changes than is the rat ovary. Too often it is assumed that, because of gross similarity, and sometimes a physiologic similarity, that those reactions occurring in one species will occur in the other species.

Summary. Two separate investigators, working in different laboratories at different times and with unrelated rat colonies have correlative data which shows, within the limits of the experiments, that spleen grafted ovaries will not noticeably extend the post adrenalectomy life of the host animal. The difference between the positive response in mice and the negative response in rats is noted. Histology of the grafted tissue was not correlated with the physiological response obtained.

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Received August 24, 1953. P.S.E B.M., 1953, v84.

Interval Between Inoculations as a Factor in Interference between Neurotropic Viruses. (20623)

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In recent studies(1,2) reported from this laboratory it was found that 3- to 4-week-old rats, which are ordinarily highly susceptible to Western equine encephalomyelitis (WEE) virus, were much less susceptible to the virus, following nasal instillation, if St. Louis encephalitis (SLE) virus had been inoculated by the same route 72 hours previously. The purpose of the studies reported here was to determine the interval that must elapse between the intranasal inoculation of SLE virus and the subsequent inoculation of WEE virus for interference to occur and also to determine how long the interfering effect persisted.

Materials and methods. The 2 viruses used have been described previously(1). Two different methods were employed in preparing the different virus suspensions used. They were prepared from virus-infected brains of 2- to 4-week-old Swiss mice by either grinding the brains in a mortar with alundum and sufficient cold, inactivated, undiluted rabbit serum to make a 10% suspension of infected brains or by homogenizing the infected brains in a Waring blendor together with sufficient of the above diluent to make a 10% suspension. The suspensions were centrifuged at about 2000 RPM for 5 minutes to sediment gross particles. The supernatant fluid was removed, distributed in 2 ml amounts in screw-capped vials and "quick-frozen" by rolling the vials in a mixture of dry-ice and alcohol. The vials were then stored in a CO_2 cabinet until needed. Virus suspensions were titrated intracerebrally in 3- to 4-week-old Swiss mice after storage for different intervals in the CO_2 cabinet. Two suspensions of each of the 2 viruses were used. One suspension of SLE virus was titrated after 5 days storage at about —70°C and the second after 11 days. The LD/50 titer(5) in each case was 1 x $10^{-8.5}$. One suspension of WEE virus was titrated 4 days after storage and the second

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* Research Paper No. 988 Journal Series, University of Arkansas.

TABLE I. Interval Required for Interference between St. Louis Encephalitis Virus and Western Equine Encephalomyelitis Virus. 18 rats in each series.

Material instilled into nose	Interval between SLE and WEE, hr	Interval between WEE and death (days)																	Results	% mortality
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		
WEE (controls)	—	0‡	0	13	4	0	0	1											18/18§	100
SLE* WEE†	2	0	0	17	1														"	"
" "	6	0	0	17	1														"	"
" "	12	0	0	16	2														"	"
" "	24	0	1	13	2	1													17/18	94
" "	48	0	0	0	4	3	0	2	0	4	2								15/18	83
" "	72	0	0	1	0	0	0	1	4	2	1	0	0	0	0	0	0	1	10/18	56
" "	96	0	0	0	0	0	0	0	3	2	1	1	1						8/18	44

* SLE = St. Louis Encephalitis virus. † WEE = Western Equine Encephalomyelitis virus.

‡ Zero indicates no deaths that day. Other number indicates No. of deaths that day.

§ Numerator indicates No. of deaths; denominator, No. of animals tested.

after 9 days. The LD/50 titers were 1 x $10^{-7.8}$ and 1 x $10^{-8.8}$, respectively. Male Sprague-Dawley rats, 24 days of age and weighing 38 to 54 g, were used. All inoculations were made under light ether anesthesia. All rats were observed daily for at least 21 days following inoculation of WEE virus.

Experimental. Interval required for interference. 144 3- to 4-week-old rats were separated into 8 groups of 18 animals each. Seven of the groups received a single intranasal inoculation of 0.05 ml of a 10% suspension of SLE virus (LD/50 8.5). At the intervals indicated in Table I these animals were challenged intranasally with 0.05 ml of a 10% suspension of WEE virus (LD/50 8.8). One group of 18 animals received WEE virus as controls. The experiments were designed so that all 144 rats were inoculated with WEE virus on the same day. The data presented in Table I show that 100% mortality was observed in the control group and also in the groups which had received SLE at either 2, 6, or 12 hours before being inoculated with WEE virus. In the group which received SLE virus 24 hours before WEE virus was inoculated, only one animal survived. It would appear that the above SLE-treated rats were as susceptible to WEE virus as were the controls, since not only was the mortality essentially the same, but in addition, they succumbed to WEE virus as early as did the controls. In the group which received WEE virus 48 hours after the inoculation of SLE virus, while only 3 of 18 animals survived, it should be noted that the survival time was somewhat longer than that in the case of the controls. It has already been pointed out that when 3- to 4-week-old rats, which receive WEE virus intranasally 72 hours after having been inoculated with SLE virus by the same route, do succumb, they survive for a longer period than do control animals(1). It was only in the rats which had been inoculated with WEE virus at 72- and 96-hour intervals that a high degree of protection was noted. The data show that roughly 50% of the animals in these 2 groups were resistant to WEE virus. This finding confirms results already reported(1,2) in which it was noted that 50 to 65% of 3- to 4-week-old rats, which were inoculated intranasally with SLE virus and challenged 72 hours later by the same route with WEE virus, resisted infection with the latter virus.

Duration of interference. The purpose of this aspect of the studies was to determine how long rats which had had SLE virus inoculated intranasally would remain resistant to WEE virus subsequently inoculated by the same route. Twenty-seven 3- to 4-week-old rats were inoculated intranasally on each of 3 consecutive days with 0.05 ml of a 10% suspension of SLE virus (LD/50 8.5). Three inoculations were given since it has been shown that 3 daily intranasal inoculations give a higher degree of protection against nasally instilled WEE virus than that obtained by a single inoculation(2). Controls of the same age received 0.05 ml of a 10% suspension of normal mouse brain (NMB) on each of 3 consecutive days at the time the test

TABLE II. Duration of Interference between St. Louis Encephalitis Virus and Western Equine Encephalomyelitis Virus.

No. of rats	Material instilled into noses of rats		Interval between NMB or SLE and WEE, days	Interval between WEE and death (days) 1	2	3	4	5	6	7	8	9	10	Results	% mortality
11	NMB* × 3	WEE	10	0	0	3	5	1	0	0	1			10/11	91
14	SLE × 3	”	10	0	0	0	0	0	1	0	1			2/14	14
11	NMB × 3	”	21	0	0	8	3							11/11	100
13	SLE × 3	”	21	0	0	0	1	2	2	0	2	2	2	11/13	85

* NMB = Normal mouse brain.

animals received their 3 inoculations of SLE virus. On the 10th day following the third inoculation of SLE virus, 14 of the SLE-treated animals received 0.05 ml of a 10% suspension of WEE virus (LD/50 7.8) intranasally. The remaining 13 SLE-treated rats were inoculated intranasally with 0.05 ml of a 10% suspension of WEE virus (LD/50 7.8) 21 days after the third inoculation of SLE virus. Eleven control animals were used for each group of test animals. The results (Table II) indicate that the interfering effect persisted for at least 10 days, since only 2 of the 14 animals proved to be susceptible to the WEE virus, while 10 of the 11 controls succumbed. The interfering effect did not persist until the 21st day, however, since rats tested 21 days after SLE virus had been inoculated intranasally were highly susceptible to WEE inoculated by the same route in that 11 of the 13 animals tested succumbed.

Discussion. From the data presented it is evident that at least 72 hours must elapse between the inoculation of the SLE virus and the subsequent inoculation of the WEE virus before a significant degree of interference can be observed. When SLE virus is instilled into the noses of normal 3- to 4-week-old rats it invades the CNS by the olfactory pathway and can be detected in the anterior rhinencephalon 24 hours later(1,3,4). However, virus at this time is present in low concentration in this part of the CNS. It would appear for nasally instilled SLE virus to interfere with the passage of WEE virus along the olfactory pathway that it must first pass along this pathway and then undergo multiplication particularly in the anterior rhinencephalon. The evidence suggests that the type of interference described here is not dependent alone upon the presence of the SLE virus in the CNS, since it has been shown that SLE virus, reaching the brain following nasal instillation, did not protect rats against WEE subsequently injected directly into the brain(1).

That the interference between SLE virus and WEE virus is not based on specific immunity is evident from data presented which show that while the interfering effect of SLE persists for at least 10 days, it cannot be demonstrated 21 days following the inoculation of the interfering virus.

Summary. St. Louis encephalitis virus, following nasal instillation, did not protect rats from Western equine encephalomyelitis virus inoculated 2, 6, 12, and 24 hours later by the same route. If the equine encephalomyelitis virus was inoculated 48 hours after the St. Louis encephalitis virus, some interference was noted since these animals survived longer than did controls. A high degree of protection against equine encephalomyelitis virus was observed in rats that had had St. Louis encephalitis virus inoculated intranasally 72 and 96 hours before the equine encephalomyelitis virus was inoculated by the same route. Rats inoculated intranasally with St. Louis encephalitis virus are resistant to nasally instilled equine encephalomyelitis virus for as long as 10 days after the former virus is inoculated, but by the 21st day this resistance disappears.

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Received August 24, 1953. P.S.E.B.M., 1953, v84.

Preliminary Observations on a New Derivative of Isoniazid (Ro 2-4969)

(20624)

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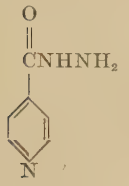
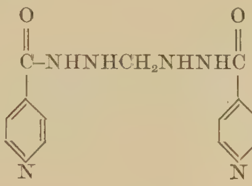
The effectiveness of isoniazid in the treatment of experimental tuberculosis in animals was first reported by Grunberg and Schnitzer(1). These investigators have since found that Ro 2-4969, a new derivative of isoniazid, has similar anti-tuberculosis properties(2). Chemically, Ro 2-4969 is 1,1-'Methylenebis (2-isonicotinylhydrazine). The structural relationship between Ro 2-4969 and isoniazid is represented as follows:



Isoniazid



Ro 2-4969

Benson *et al.*(3) found that the subacute toxicity of Ro 2-4969 in rats is less than that of isoniazid; acute toxicity in other species also compares favorably with that of isoniazid. According to Schnitzer and Grunberg(2), the chemotherapeutic ratio (LD_{50}/PD_{50}) of Ro 2-4969 in mice is greater than 260; the comparative value for isoniazid is 44. In view of these promising reports the present study was undertaken to determine the effects of Ro 2-4969* on tuberculosis in man. Particular attention was paid to the absorption of Ro 2-4969 because of its low solubility.

Methods. The concentration of Ro 2-4969 in blood plasma was determined according to the method of Rubin *et al.*(4). This technic is based on the determination of isonicotinic acid. All results of determinations of plasma levels are therefore expressed as micrograms of isonicotinic acid per ml. It is not known in what form Ro 2-4969 exists in the blood. Most of the subjects used in this study were patients at the Lakeview Sanatorium. We are indebted to Dr. John K. Shumate, Superintendent and Medical Director of Lakeview, for his generous cooperation and assistance in the care and study of these patients.

Results. Fig. 1 presents the plasma concentrations following a single oral dose of Ro 2-4969 compared with a similar dose of isoniazid in the same subject. The rapid rise and decline following isoniazid is typical of the drug. No detectable concentration was found 24 hours after the administration of isoniazid. Ro 2-4969, on the other hand, reached its peak more slowly, a significant level was present at the end of 12 hours and a detectable concentration at the end of 24 hours.

The presence of discernible quantities at the end of 24 hours led to an examination of the possible accumulation of Ro 2-4969 if it were given over a period of several days. The plasma concentrations of 3 patients who were given the drug daily for 8 days were observed. Ro 2-4969 was administered for the first 4 days at a total dose of 5 mg/kg/day and then increased to 10 mg/kg/day for the remaining 4 days. The divided doses were given at 8:00 A.M., noon, and 5:00 P.M.

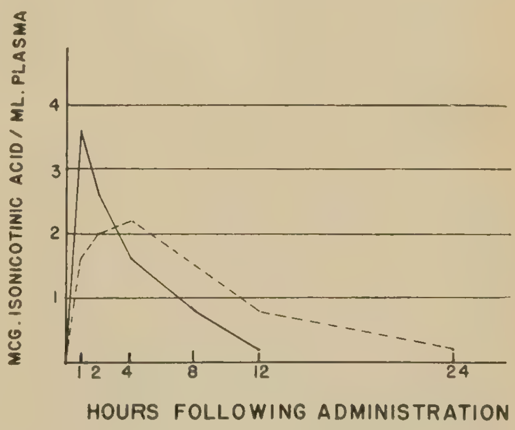


FIG. 1. Plasma concentrations in the same subject following oral administration of 200 mg each isoniazid (solid line) and Ro 2-4969 (broken line).

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* Made available for this study by Dr. M. J. Schiffrin, Hoffmann-La Roche, Inc., Nutley, N. J.

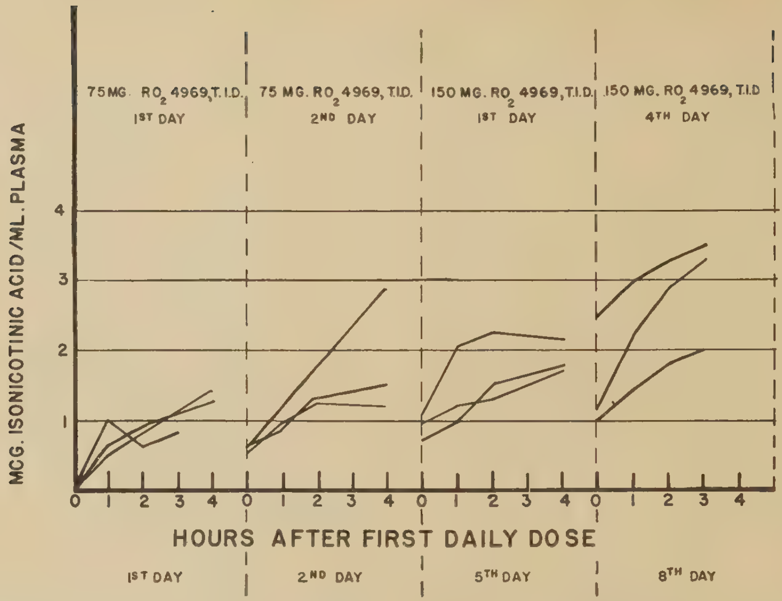

FIG. 2. Plasma concentrations in 3 subjects receiving Ro 2-4969 daily in divided doses for a period of 8 consecutive days. Figures at top refer to day of administration at either the 75 mg or 150 mg level. Days listed at bottom of figure refer to the experimental days.

Plasma levels were determined before the morning administration and at one, 2, and 4 hours after the first dose. A definite cumulative tendency was observed throughout the 8-day period (Fig. 2).

The possibility of accumulation over longer periods was studied in 10 patients who received a single daily dose of Ro 2-4969, 10 mg/kg, over a period of 9 weeks. Plasma concentrations were determined once weekly just prior to the administration of the daily dose, hence 24 hours following the previous dose. These data are presented in Table I. There was a definite tendency toward establishment of a continuous blood concentration at a high therapeutic average level of 1.0-1.5 μg/ml plasma from the third week on. In addition to these 10 patients, 16 patients were given the same dosage but without regular determinations of plasma concentrations.

Discussion. It is evident that when Ro 2-4969 is administered orally, a derivative of isonicotinic acid appears in the plasma. The peak concentration appears later and is lower than that following the administration of isoniazid; however, measurable quantities persist longer than with isoniazid.

At the 10 mg/kg/day dosage, there is a cumulative effect even when Ro 2-4969 is given as a single daily dose, so that after 2 or 3 weeks clinically significant concentrations (one or more μg/ml) are present 24 hours following each dose. It is reasonable to assume that such significant levels persist throughout the entire 24-hour period.

A detailed report of the clinical observations made during the treatment of these patients will appear subsequently. In general, the clinical, x-ray and bacteriological response in 26 patients treated for 2 months or longer have paralleled those seen with isoniazid therapy. As with any new agent, the final assessment of the value of Ro

TABLE I. μg Isonicotinic Acid per ml Plasma.

	Weeks of treatment							
	1	2	3	4	5	6	7	8
B.	.2	1.0	1.1	1.0	.8	1.6	.8	.8
C.	.6	1.0	1.2	1.4	1.5	1.1	—	1.1
G.	—	.6	.8	.8	.9	.9	—	1.1
H.	.5	1.3	1.9	—	2.5	1.1	—	1.6
W.	.6	1.0	1.6	—	1.7	1.4	—	—
A.W.	.6	.7	.9	1.1	1.3	1.4	1.6	1.2
J.B.	.3	1.2	.8	—	1.9	.9	1.2	1.2
F.	.5	.8	.8	1.2	1.6	1.1	—	1.1
S.	.7	.7	.8	—	1.4	.8	—	—
K.	.4	1.0	—	1.1	.9	—	1.7	.8
Avg	.5	.9	1.1	1.1	1.5	1.1	1.3	1.1

2-4969 in the treatment of human tuberculosis must await prolonged clinical trial. It is significant, however, that no toxic reactions to Ro 2-4969 were observed.

Summary. 1. Ro 2-4969, a compound of low solubility, related to isoniazid, has been administered experimentally to human subjects to determine the rate and extent of absorption from the gastrointestinal tract. 2. The compound or its derivatives appeared in the plasma and, probably because of slow absorption, detectable and clinically significant amounts remained for a longer period than when the highly soluble isoniazid was administered. 3. The drug administered in a single daily dose of 10 mg/kg/day was found to maintain drug concentrations of one μg/ml of plasma without dangerous cumulative effect. 4. No toxic reactions have been observed in 26 patients treated for 2 months or more.

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Received August 24, 1953. P.S.E.B.M., 1953, v84.

The Effect of Adrenalectomy on Propulsive Motility of the Small Intestine in Rats. (20625)

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Digestive disturbances occur in Addison's disease and in adrenal insufficiency in animals (1,2), but relatively few direct studies of gastro-intestinal behavior after adrenalectomy have been reported. Anorexia, nausea, vomiting, either constipation or diarrhea, and abdominal pain are symptoms in Addison's disease; congestion, diffuse hemorrhage of the intestine and bloody diarrhea are common in adrenalectomized animals. Vogt(3) found that the circular muscle coat of the isolated intestine of the adrenalectomized rabbit frequently developed powerful, spasmotic, rhythmic contractions, while Auerbach's plexus often failed to respond to normally effective stimuli. Since adrenal extract did not restore normal activity, it was concluded that the abnormal intestinal behavior was due to secondary effects of adrenal removal.

Methods. Propulsive motility of the small intestine was studied by the method of Van Liere *et al.*(4) in 82 young male, albino Wistar rats, weighing from 90 to 150 g. Thirty adrenalectomized rats were tested 4 to 7 days after operation. Since two types of control animals (intact and sham-operated) are necessary, 15 intact and 19 sham-operated were tested. Purina Laboratory Chow and tap water was supplied *ad libitum*, and daily food intake was measured. Since adrenalectomized rats lose their appetite, controls for the effect of relative starvation were performed by feeding an additional 6 intact and 12 sham-operated rats a daily amount of food equal to the average amount eaten by adrenalectomized rats and testing them after periods of reduced food intake equal to that after adrenalectomy. In all groups, the rats were starved for 24 hours prior to testing for intestinal propulsion.

Results. The results are presented in Table I as percent of the total number of rats in each experimental group falling within each 5% range of the length of the small intestine traversed by the test meal of charcoal. No essential difference occurred between the intact and sham-operated rats. Partial starvation of both normal and sham-operated rats produced some increase in intestinal motility. However, no significant differences were found

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* Many of the experiments were performed by the following graduate students: Milton Stern, Harold Carlin, and Mario M. Boschetti.

TABLE I. Effect of Adrenalectomy or Relative Starvation on Propulsive Motility of Small Intestine in Rats (Expressed as % of Total No. of Rats in Each Experimental Group).

		% of small intestine traversed by test meal								
Type of rat	No. of rats in group	56–60	61–65	66–70	71–75	76–80	81–85	86–90	91–95	96–100
Intact normal	15			13*	20	27	20	20		
Normal starved	6					33	17	33	17	
Sham-operated	19		5	11	11	21	31	21		
Sham-operated starved	12		8		8	25	34	8	17	
Adrenalectomized	30	10	3	3		10	7	23	34	10
Total controls	52		4	8	11	25	27	19	6	

* % given as the nearest whole figure.

among the 4 control groups, and consequently the combined data were used for comparison with the adrenalectomized group.

The adrenalectomized rats differed in two respects from the controls. The small intestines were more active, since 67% exhibited propulsion of the charcoal beyond 85% of the intestinal length. However, 10% had less active intestines than the controls, propelling the charcoal less than 61% of the distance.

The altered propulsive activity of the intestine was not related to the physiological condition of the adrenalectomized rats. For example, severe adrenal insufficiency appeared in approximately one third of those with increased motility and also in the same proportion showing decreased motility. The condition of the other two-thirds in each group varied from poor to fair or good.

Discussion. The increased motility of the small intestine in adrenalectomized rats may be associated with changes in the electrolyte balance in the body. Hyperpotassemia occurs during adrenal insufficiency in rats(5,6) and high potassium may stimulate smooth muscle (7,8). Furthermore, hypomotility occurs in rats during potassium deficiency and potassium injections stimulate active movements(9).

Summary. Adrenalectomized rats showed greater propulsive motility of the small intestine than intact, sham-operated, or partially starved controls. A small proportion exhibited abnormally reduced activity. The altered activity during adrenal insufficiency may be associated with changes in the potassium balance.

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Received August 25, 1953. P.S.E.B.M., 1953, v84.

Adjuvant Action of Phosphorylated Hesperidin. (20626)

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Experimental attempts to stimulate antibody formation and to promote the persistence of an effective titer have been undertaken ever since Pasteur and Joubert published their work in 1877. Since that time a sizeable body of information has accumulated in the literature on the use of adjuvants of widely diverse natures as exemplified by reports of the activities of various chemical agents, foreign proteins and non-related bacteria. Perhaps the most widely investigated adjuvant is that which consists of water and mineral oil with an emulsifying agent. Successful results with this adjuvant have been ascribed to "the slow prolonged absorption of the active antigen"(1) or "to the direct contact of the oil with the tissues eliciting the gathering of phagocytes that not only attempt to carry off the oil but are also effective in antibody formation"(2).

It has been shown that hyaluronidase can be effectively inhibited *in vitro*(3) and *in vivo*(4) by phosphorylated hesperidin. Application of this principle has been extended to experiments designed to determine if this hesperidin derivative will delay absorption of parenterally administered substances. Work in this laboratory has proven, in fact, that trypsin(5), heparin(6), vitamins, antibiotics, pressor amines, and local anesthetics(7) have a more prolonged blood level when administered with phosphorylated hesperidin. If a delay in absorption of a particulate antigen from the injection site will produce an adjuvant action, then the addition of a compound such as phosphorylated hesperidin to a vaccine should be attended by an increase and prolongation of antibody titer. Verification of this has been achieved in animal experiments as follows:

Three groups of 6 rabbits each were immunized with single subcutaneous injections of formalinized typhoid organisms suspended in a different medium for each group. The

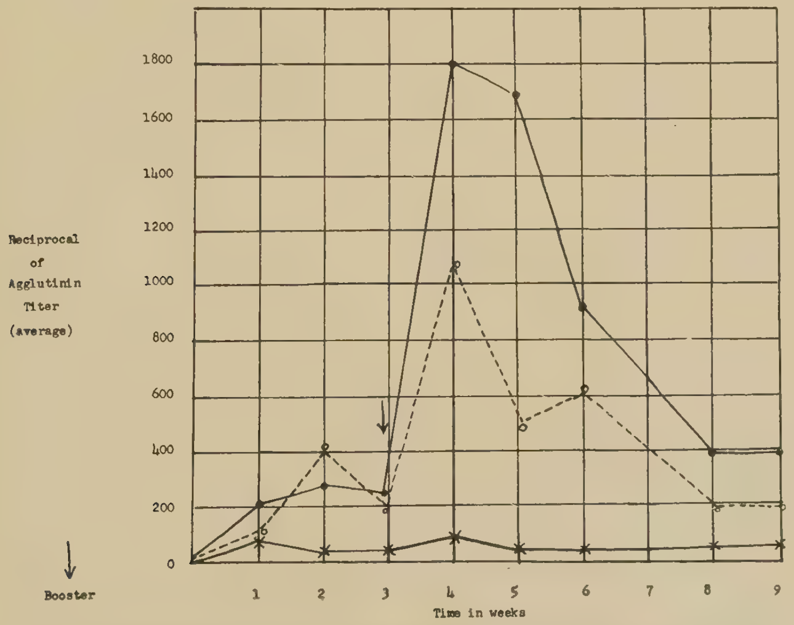

FIG. 1. Graph showing antibody titer in rabbits after administration of antigen in various vehicles. —×— Typhoid in saline. —●— Typhoid in 4% phosphorylated hesperidin. ---○--- Typhoid in water in oil emulsion.

suspending media were saline, 4% phosphorylated hesperidin solution and a water-in-oil emulsion. In each case the amount injected consisted of one cc of material containing 500 million organisms/cc. The water-in-oil emulsion was composed of 2 parts saline suspension of organisms, 2 parts paraffin oil and one part G 1096 Atlas emulsifier. A booster injection was given at the end of the 3rd week. Blood samples were collected each week and the antibody titration carried out by means of the standard agglutination test.

The results are pictured graphically in Fig. 1 which shows the unusual effectiveness of phosphorylated hesperidin as an adjuvant. Figures are averages for the 6 rabbits in each group. In view of the current interest in gamma globulin for passive immunization against poliomyelitis, and the relative shortness of the period of effectiveness, it is intended to determine if the use of phosphorylated hesperidin might extend the period of protection now offered by gamma globulin.

Summary. An adjuvant action of phosphorylated hesperidin in the induction of the antibody response to typhoid vaccine has been demonstrated.

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Received August 25, 1953. P.S.E.B.M., 1953, v84.

Influence of Low Tryptophan Diets Containing 6-Methyltryptophan on Oral Infection with Poliomyelitis Virus.[*] (20627)

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A study of the relation of the nutritional state of the host to susceptibility to Lansing (Type II) poliomyelitis in mice has established the fact that the development of Lansing infections is altered in animals suffering from a number of nutritional deficiencies (1). Tryptophan deficiency is particularly effective in modifying poliomyelitis in mice (2,3) and mice may be partially protected from Lansing virus by feeding the tryptophan analogue, 6-methyltryptophan(4). Preliminary experiments indicating that low tryptophan diets containing 6-methyltryptophan also exert some protective action against oral infection with poliomyelitis virus in monkeys are described here.

Toxicity of 6-methyltryptophan. Our studies in mice(4,5) had shown that the greatest protection against Lansing infections was observed in the animals fed diets containing a small amount of tryptophan (0.05%) and 10 times that amount of 6-methyltryptophan. The tolerance of 1.2 to 2.2 kg Philippine cynomolgus monkeys[‡] for 6-methyltryptophan[§] (6-MT) was approximated by feeding 6-MT in a diet consisting of: Corn grits,[‖] 89%; corn oil[¶] (fortified with oleum percomorphum[**] 25 ml/liter), 5.5%; Salts "IV" of Phillips and Hart(6), 4%; 1-lysine HCl, 1.0%; and DL methionine, 0.5%. Water soluble vitamins

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[*] Aided by a grant from the National Foundation for Infantile Paralysis.

[†] Present address: Department of Infectious Diseases, University of California Medical School, Los Angeles.

[‡] Obtained from Okatie Farms.

[§] We are greatly indebted to Charles Pfizer and Co., for the 6-methyltryptophan which they generously provided.

[‖] Quaker "Hominy Grits".

[¶] Mazola.

[**] Mead Johnson and Co.

TABLE I. Summary of Experiments Showing Influence of Diets Containing 6-Methyltryptophan (6-MT) on Susceptibility of Cynomolgus Monkeys to Oral Infection with Poliomyelitis.

Exp. 1						Exp. 2						Exp. 3					
2% 6-MT, 0.05% tryptophan in corn grit basal						2% 6-MT in corn grit basal						2% 6-MT in corn grit basal					
6-MT			M-3 control			6-MT			M-3 control			6-MT			M-3 control		
No.*	F†	P‡	No.	F	P	No.	F	P	No.	F	P	No.	F	P	No.	F	P
1	0	8	17	5	6	31	5	7	37	6	7‖	57	7	0¶	67	3	10
6	0	8	24	4	7	32	3	0	43	6	8	51	6	0	62	9	12
11	0	8	16	5	8	25	0	0	36	5	8	53	6	0	64	12	13
8	7	9	21	7	8	26	0	0	41	9	10	49	0	0	63	3	30
15	9	9	20	8	8	27	0	0	38	6	11	50	0	0	59	3	0
9	9	10	18	7	9	28	0	0	42	7	12	52	0	0	68	4	0
13	0	10	22	8	9	29	0	0	35	0	14	54	0	0	66	6	0
3	0	0§	23	8	9	30	0	0	44	0	19	55	0	0	61	0	0
4	0	0§	19	8	11	33	0	0	39	0	0	56	0	0	65	0	0
						34	0	0	40	0	0	58	0	0			
Total paralysis		7			9			1			8			0			4

* Monkey No.

† F = Days after first virus feeding on which fever of 103°F or higher developed.

‡ P = " " " " when paralysis appeared.

§ Monkeys No. 3 and No. 4 resisted intracerebral challenge with 500 LD_{50} Wisconsin '45 virus 6 weeks after oral inoculation. All other surviving animals developed typical poliomyelitis following intracerebral challenge.

‖ Monkey No. 37 was found dead on the 7th day. Examination of the brain and cord revealed typical histopathology of severe poliomyelitis.

¶ Monkey No. 57 was slow, dull and ataxic 9 days after inoculation with virus but recovered without developing paralysis.

were provided as a supplement in the drinking water each morning(7). Control animals were fed our basal ration (M-3) containing 18% casein(7). The addition of 2% 6-MT to the corn grit basal, either with 0.05-0.1% supplementary tryptophan or without had no consistent adverse influence, as compared to the corn grit basal alone. Some animals lost weight rapidly on these rations, but so also did animals on the corn grit basal without 6-MT; and occasional animals failed to thrive even on 18% casein.

Subsequent nutritional observations were limited to those animals which also received virus (Exp. 1, 2 and 3, Table I) and definite conclusions as to the chronic toxicity of 6-MT cannot be drawn. It would appear that, as has been shown to be true in the mouse(5), 6-MT is not a highly active nutritional antagonist for tryptophan in the monkey. Several of the monkeys in Exp. 2 and 3 (Table I) developed anorexia during the last days of the experiment and it is thought that this may have been a manifestation of chronic tryptophan deficiency. On the other hand, it is probable that some animals would tolerate 2% 6-MT in the corn grit basal for a longer time and that concentrations considerably greater than 2% could be fed for shorter periods. Although a general tendency to diarrhea was observed among all of the monkeys including those receiving 18% casein, none developed the severe dysentery characteristic of deficiencies of folic acid and other water soluble vitamins.

Influence of 6-MT on oral infection. Philippine cynomolgus monkeys weighing from 1,200 to 2,200 g and the Wisconsin '45 strain of Type I poliomyelitis virus in the form of a pool of 10% monkey brain-cord suspension, prepared and stored in 50 ml aliquots at –40°C, were employed. The monkeys were inoculated by offering them 20 ml of virus suspension, sweetened with sucrose, in their drinking cups. Water was withheld on the day or days virus was given until the virus suspension was consumed. Ordinarily, the animals drank the virus suspension immediately. The indicated amounts of 6-MT and tryptophan were added to the dry corn grit basal, moistened with water and cooked in the autoclave for 30 minutes at two pounds pressure. The resulting ration was granular but soft and was much more palatable to the monkeys than was a dry ration. The ration was offered in small amounts, about 50 g, 3 times daily. Control animals received the 18% casein basal(7).

In the first experiment, a group of 9 monkeys was placed on the basal ration containing 2% 6-MT and 0.05% dl-tryptophan for 7 days; and on the 8th, 9th, and 10th days this group and a control group of 9 monkeys were fed a total of 120 ml of 10% virus suspension (about 1,200,000 intracerebral LD_{50}) in 6 doses of 20 ml each. Temperatures were recorded daily and the animals were observed for signs of disease twice daily. Three weeks after the first virus feeding the surviving animals were placed on the 18% casein basal diet. The results are recorded in Table I.

Exp. 2 and 3 (Table I) were similar except that 1) the 2% 6-MT ration was not supplemented with tryptophan and 2) the monkeys received two feedings of 20 ml of a 1:400 dilution (approximately 10,000 intracerebral LD_{50}) from the same pool of Wisconsin '45 virus on the eighth day of the experiment. The surviving animals of all 3 experiments were challenged intracerebrally with 500 LD_{50} Wisconsin '45 virus 6-9 weeks after their oral exposure.

In Exp. 1, there was a suggestion of protection against the virus in the 6-MT group, *i.e.*, incubation periods were longer, febrile prodromata less pronounced and two animals escaped paralytic infection. These 2 animals, No. 3 and 4, apparently did experience an inapparent infection for they resisted a large intracerebral challenge 9 weeks later. In the second and third experiments, the smaller inoculum resulted in a lower incidence of paralytic infection in the controls, 8/10 and 4/9 respectively, while only one of the 20 animals receiving 6-MT became paralyzed. The surviving animals in the latter experiment succumbed to intracerebral challenge.

Summary. These experiments are obviously of a preliminary nature but they do show that the susceptibility of a primate to poliomyelitis via a natural portal of entry may be altered and they suggest that this approach to the modification of susceptibility to poliomyelitis should be explored further.

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Received August 31, 1953. P.S.E.B.M., 1953, v84.

Effect of Adrenocortical Steroids on Duration of Pentothal Anesthesia in Adrenalectomized Mice. (20628)

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It is well known that the adrenalectomized animal becomes less resistant to various types of damaging agents and more sensitive to various drugs, including anesthetics. And recently, much has been published on the central action of adrenocortical steroids. It is supposed from these that the adrenal gland may play an important role in anesthesia. The present experiment was performed to confirm this.

Materials and methods. The animals used in this study were 60 female albino mice weighing about 20 g, divided into 6 groups of 10 mice each, as shown in Table I. The

TABLE I. Duration of Pentothal Anesthesia in 6 Groups of Albino Mice.

Operation	Treatment	Mean waking time (min.)	Index of waking time	P (%)
Intact	—	25	1.0	
Adrex	—	110	4.4	$P < 0.5$
"	Cortisone acetate	33	1.3	$25 < P < 50$
"	Hydrocortisone acetate	28	1.1	$50 < P$
"	DOCA	100	4.0	$P = 0.5$
"	Saline	83	3.3	$P < 0.5$

anesthetic employed was pentothal sodium (Ravonal Tanabe). Cortisone acetate and hydrocortisone acetate, both in saline suspension (Cortone and Hydrocortone* Merck), and DOCA in sesame oil (Cortate Schering) were administered subcutaneously. The dose of the steroids was 0.5 mg daily for 2 days; 0.3 mg/10 g of pentothal sodium (3 mg/ml in isotonic saline) was administered intraperitoneally about 18 hours after the last injection of the steroids (about 48 hours after adrenalectomy). The anesthesia time was determined by Winter's method(1) on the same number of animals from each group at one time to avoid day to day variation. By Snedecor's t-test, the difference in mean anesthesia time between groups was determined as significant if p was smaller than 5%.

Results. As shown in Table I and Fig. 1, the mean duration of pentothal anesthesia in the adrenalectomized group is 4.4 times longer than in the intact group ($p < 0.5\%$). In the adrenalectomized groups treated with cortisone acetate or hydrocortisone acetate, anesthesia is not significantly longer than in the intact group—1.3 times ($25\% < p < 50\%$) and 1.1 times ($50\% < p$), respectively, but is significantly shorter than in the adrenalectomized non-treated group ($0.5\% < p < 1\%$). The difference between the cortisone acetate and hydrocortisone acetate treated adrenalectomized groups is not significant ($25\% < p < 50\%$). The anesthesia time of the adrenalectomized DOCA treated group is 4.0 times longer than that of the intact group ($p = 0.5\%$), but not significantly different from that of the adrenalectomized non-treated group ($50\% < p$). That of the adrenalectomized saline treated is 3.3 times longer than that of the intact ($p < 0.5\%$), but not significantly different from that of the adrenalectomized non-treated ($25\% < p < 50\%$).

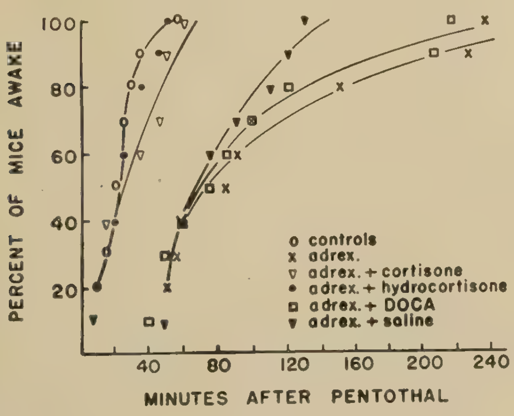

FIG. 1. Effect of adrenocortical steroids upon the waking response curve of adrenalectomized mice treated with pentothal sodium.

Discussion. Prolongation of the duration of pentothal anesthesia in adrenalectomized mice is considered to have been caused mainly by the deficiency of glucocorticoids, because the anesthesia time almost returned to the normal level when the animals were treated with cortisone acetate or hydrocortisone acetate, but not with DOCA.

Winter(2) reported that cortisone but not DOCA antagonizes the effect of hexobarbital (Evipal) in normal mice. By the present experiment, it is shown that these steroids act in the same manner upon the effect of pentothal sodium in adrenalectomized mice.

Testing the effect of adrenocortical steroids on the electro-shock seizure threshold (EST) of rats, Woodbury(3) demonstrated that cortisone acetate was more potent in central stimulating action than hydrocortisone acetate. In the present experiment, however, no significant difference was found between these 2 steroids at the dose levels employed. Further, Woodbury(4) has shown that chronic administration of diphenylhydantoin or phenobarbital with DOCA pellets caused an increase in the EST greater than the sum of the increases produced by the 2 drugs given separately. And so, it might be expected that treatment with DOCA would also prolong the anesthesia time in adrenalectomized mice. However, such an effect was not demonstrated.

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* We thank Dr. C. A. Winter of Merck Institute, Rahway, N. J., for supplying us with Cortone and Hydrocortone.

Summary. The effects of the administration of cortisone acetate, hydrocortisone acetate and DOCA on the duration of pentothal anesthesia in adrenalectomized female mice were ascertained. Adrenalectomy induced a highly significant prolongation in the duration of pentothal anesthesia. Pretreatment of cortisone acetate and hydrocortisone acetate returned the anesthesia time to normal level in adrenalectomized mice, while DOCA and saline had no effect.

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Received October 5, 1953. P.S.E.B.M., 1953, v84.

Sulfate Fixation by Embryonic Chick Lung in Tissue Culture.[*] (20629)

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The authors have studied the synthesis of protein, nucleic acid, and collagen in chick embryonic tissue by a quantitative roller tube method(1-3). In view of the relationship of chondroitin to collagen, it was considered worth while to study the uptake of S^{35}-labeled sulfate under these conditions. Layton(4-7) has found a high rate of sulfate fixation after 45 hours' incubation with embryonic chick heart, skeletal muscle, liver, and bone in Tyrode's solution containing labeled sulfate. He suggested that the labeled sulfate was bound in the chondroitin sulfate of the connective tissue ground substance; cortisone added to the medium inhibited sulfate fixation by heart and skeletal muscle.

Methods. Roller tube cultures of 15-day chick lung were prepared as previously described(1) using 20-40 mg of tissue per tube. Tyrode's solution and 25% chick embryo extract were used as media. The medium was changed 3 times weekly, and incubation of the cultures were continued in embryo extract for as long as 6 weeks at 37°C. At the termination of incubation, the medium was poured off, the tissue was washed with water, and the tissue fragments were rubbed from the walls of the tubes in 5% trichloroacetic acid. The contents of 4-5 tubes were pooled, dehydrated, and delipidized. Details of the procedures have been described previously (1). In order to determine the incorporation of labeled sulfate into the tissue, sulfate[§] was added to the nutrient medium in quantities of 0.002 millicuries per ml of medium. Uptake of the labeled sulfate was recorded as counts per minute per milligram[‖] of the lipid-free

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[*] Aided in part by a grant from the Central Scientific Fund of the College of Medicine of the State University of Iowa. Presented in part at the meeting of the Tissue Culture Assn. at Columbus, Mar. 1953.

[†] Postdoctoral Fellow of the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

[‡] Present address: Medical Research Division, Standard Oil Development Co., Linden, N. J.

[§] The radioactive sulfate used in this investigation was supplied by the Oak Ridge National Laboratory on allocation by the U. S. Atomic Energy Commission.

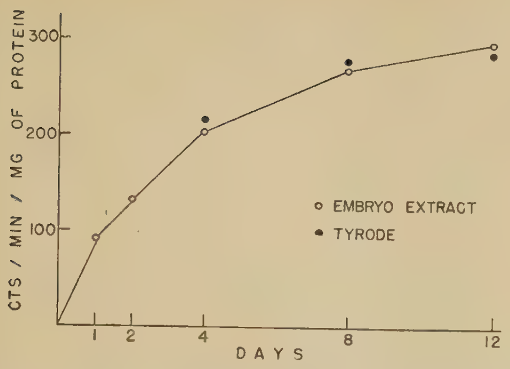

FIG. 1. Incorporation of S^{35}-labeled sulfate into protein of lung cultures in 25% embryo extract and in Tyrode's solution.

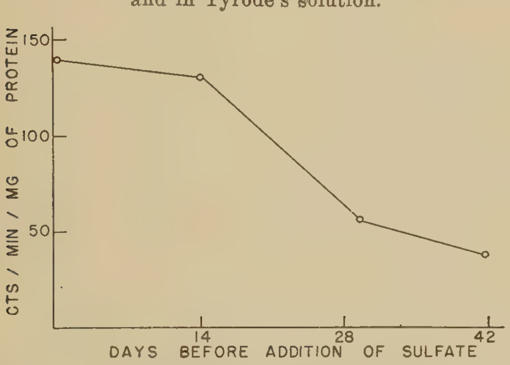

FIG. 2. Incorporation of S^{35}-labeled sulfate into protein of lung cultivated in 25% embryo extract for varying times prior to addition of the labeled sulfate. Incubation in presence of labeled sulfate continued for 2 days.

dry tissue; corrections for decay and self-absorption were made.

Results. Fig. 1 presents data showing the incorporation of radioactive sulfate into lung tissue cultivated in 25% embryo extract and in Tyrode's solution. An initial rapid uptake is followed after 8 days by a slower rate of incorporation in both media.

Tissues incubated in embryo extract at 4°C did not take up the S^{35}. There was no incorporation of the sulfate in lung cultivated in media containing 0.0005 M dinitrophenol, 0.005 M fluoride, 0.001 M cyanide, or 0.001 M azide, all of which inhibited proliferation of the tissue. There was no uptake of the sulfate in embryo extract or in plasma protein at either 37°C or 4°C in the absence of tissue.

The incorporation of sulfate into cultivated lung tissue of varying ages is shown in Fig. 2. Roller tube cultures of lung were grown in 25% embryo extract for varying times up to 42 days; medium containing labeled sulfate was added for the final 2 days of cultivation only. After 2 weeks the uptake of sulfate decreased very rapidly, bearing out previous observations(2,3) that the numbers of active cells decrease after a 2-week period, while there is a concomitant increase of extracellular protein.

The addition of 50 μg/ml of cortisone acetate¶ to the embryo extract medium did not change appreciably the incorporation of sulfate into the tissue (Fig. 3), even though a slight inhibition of the proliferation of the tissue fragments occurred for a 2-day period initially. The use of tissue from cortisone-treated embryos(3) presented similar results when cultivated in a medium containing cortisone, although here the initial inhibition of growth was prolonged to 8-10 days.

Summary. Chick embryonic lung tissue cultivated *in vitro* in the presence of sulfate labeled with S^{35} fixed sulfate in the protein of the tissue. Lung tissue which was not proliferating did not incorporate sulfate. Cultures of lung older than 2 weeks had a greatly reduced ability to fix sulfate. The presence of cortisone in the medium did not alter the incorporation of sulfate into the tissue.

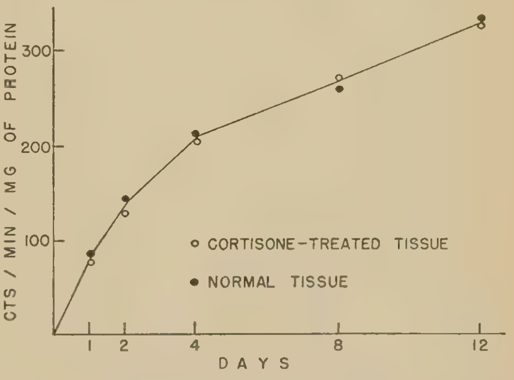

FIG. 3. Incorporation of S^{35}-labeled sulfate into normal and cortisone-treated lung cultivated in presence of cortisone acetate (50 μg per ml of the medium).

|| The authors wish to express appreciation to the Department of Physiology for the use of the Geiger-Müller counter.

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Received October 7, 1953. P.S.E.B.M., 1953, v74.

Beneficial Effects of Cortisone on Survival of Rats Infected with *D. pneumoniae*. (20630)

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It has been reported that acute bacterial infections result in adrenal enlargement suggestive of increased cortical function(1). It has also been demonstrated that adrenalectomy causes an increased sensitivity to a wide variety of stresses including that of acute infection(2). A number of investigators have shown that adrenalcortical extracts and 11-oxygenated steroids can restore the resistance of adrenalectomized animals to non-specific stress. Only a few reports, however, are available to indicate that cortical hormones can counteract the stress of acute bacterial infections either in adrenalectomized or intact animals(3,4). All previous evidence, on the contrary, suggests that massive doses of cortisone lower the resistance of both adrenalectomized and intact animals to infection(5-8). The data presented in this paper demonstrate that there is an optimal dose of cortisone which can enhance the resistance and survival of both adrenalectomized and intact rats infected with *Diplococcus pneumoniae*.

Methods and material. The rats used in this study were the MS-2 strain obtained from Manor Farms, Staatsburg, N. Y. They were fed a standard mixed natural food diet *ad libitum* and were housed in an air-conditioned animal room. The animals were infected intradermally with 0.1 cc of a 1 x 10^{-6} or 1 x 10^{-7} dilution of a 6 hour culture of *D. pneumoniae*, Type I. Each injection contained approximately 17 to 250 organisms as determined by plate counts on 5% blood in brain-heart agar. Blood samples for bacteremia studies were obtained either by a direct heart puncture or from tail blood collected in a pipette. Adrenalectomized animals were operated at least 5 days prior to infection and were maintained on 1.0% saline. Cortisone acetate (Cortone Merck) was administered subcutaneously as a micro-crystalline suspension in a single daily dose. The hormone injections were initiated 5 days before infection and the animals were treated and observed for a 10-day period following the infection. A total of 545 animals were used in this study of which 90 were adrenalectomized controls, 195 adrenalectomized treated, 115 intact controls, and 145 intact treated.

Results. It can be seen from Fig. 1 that adrenalectomy markedly decreased the survival rate of rats infected with *D. pneumoniae*. Cortisone, in doses ranging from 2.5 to 5.0 mg/rat/day, significantly increased the survival of both the adrenalectomized rats and the rats with intact adrenals. Maximal sur-

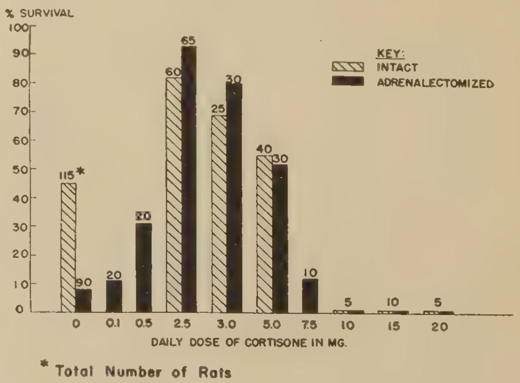

FIG. 1. Effect of Varied Doses of Cortisone on Survival of Rats Infected with *D. pneumoniae*.

TABLE I. Blood Culture Studies of Rats Receiving Cortisone 48 Hours Post Infection.

	Mean No. of pneumococci per cc of blood			
Daily dose (mg)	No. of animals	Adrenalectomized	No. of animals	Intact
0	40	14200 ± 3000*	59	9200 ± 4000
2.5	39	1100 ± 650	25	1600 ± 1100
15.0	19	240000 ± 155240	15	102500 ± 27821

* Stand. error.

vival occurred in both groups at the 2.5 mg dosage level. The resistance of adrenalectomized and intact rats was reduced when cortisone was given in doses of 10-20 mg/rat/day and the animals died with overwhelming pneumococcal sepsis. Blood culture studies revealed that the bacteremia was minimal at the 2.5 mg level in both the adrenalectomized and intact control groups (Table I).

Discussion. It is clear from the present study that under certain conditions, cortisone may significantly enhance host resistance to infection in both intact and adrenalectomized rats. The manner in which cortisone increases the resistance of the rat to infection is not known at present. However, several observations are of interest in speculating about the mechanisms concerned with the beneficial effects of intermediate doses of cortisone. First, the adrenal glands enlarge during infection and secrete increased amounts of adrenal cortical hormones, as judged by the fall in circulating eosinophiles, thymic atrophy, and the reduction of adrenal ascorbic acid and cholesterol. This response is presumed to be an attempt on the part of the host to counteract the stress of acute infection. The question arises as to whether or not the adrenal cortices are able to secrete sufficient quantities of hormone to meet the requirement of the host during severe infections. If not, perhaps additional amounts of hormone would aid in maintaining the defense mechanism at maximal efficiency. This view is supported by the results of the survival and blood culture studies reported in the present paper which show a maximal survival and minimal bacteremia at the optimal cortisone level of 2.5 mg/rat.

A second fact which may be of interest in regard to the possible mechanism of action in cortisone on infection is the ability of the hormone and the adrenal cortical extracts to increase the phagocytosis of particulate matter by the fixed histiocytes of the reticulo-endothelial system(9-11). It should be emphasized, however, that these reports have dealt with the phagocytosis of thorium dioxide, India ink, and carboxy cellulose. Their relationship to our studies with pneumococci is at present only speculative.

A third factor of possible significance is the action of cortisone in counteracting the effects of certain toxins. Cortisone has been reported to produce a sense of well-being in patients critically ill with pneumococcal pneumonia (12) and in animals to counteract the lethal effect of toxins and pyrogens(13,14). Thus, doses of cortisone sufficient to offset partially or completely the toxemia associated with the infection, but not great enough to suppress the inflammatory reaction or other important defense mechanisms, may be an important factor in enhancing the survival of animals suffering from acute bacterial infection. This would seem to be of particular importance in view of the marked susceptibility of adrenalectomized animals to toxins and noxious agents in general. It may also play an equally significant role in animals with intact adrenals.

It should be emphasized that at present there is no method available for determining the optimal requirement of cortisone in any given infection or stress. It is apparent that the optimal dose will vary with numerous factors including the animal species, the infectious agent, the pathology of the lesion, and the duration of the stress. A dose of 2.5 mg of cortisone may be "pharmacological" when administered to a normal, noninfected rat while the same dose may be within the "physiological" range when given to an infected animal.

Summary. The data demonstrate that cortisone in the dosage range of 2.5-5.0 mg/day can enhance the survival of intact and adrenalectomized rats subjected to the stress of experimental pneumococcal infection. The results of bacteremia studies suggest that optimal doses of cortisone operate by enhancing host resistance to the infectious agent. On the other hand, doses larger than 5 mg per rat per day lower the resistance of rats to this infection. Hence, in cortisone therapy, as in the case with certain other hormones, *optimum dosage* is important and this may vary with different conditions.

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Received September 4, 1953. P.S.E.B.M., 1953, v84.

Properties of Bovine Anti-Hemophilic Factor. (20631)

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The production of a purified and concentrated preparation of human anti-hemophilic factor (AHF) has been hampered by the marked instability of this substance. Such rapid deterioration of plasma AHF occurs with storage either at room or refrigerator temperature, that more than half the activity may be lost within 24 hours. Moreover, AHF is largely destroyed in the process of plasma fractionation. Cohn's fraction I is the most potent plasma derivative presently available; yet various lots of this preparation contain only 8-35% of the anti-hemophilic activity represented in fresh normal plasma(1).

Although some success has been reported in the stabilization of human AHF(2), another approach would be the preparation of an intrinsically stable AHF derived from animal sources. Bovine plasma is known to possess anti-hemophilic activity, and potent fractions have been prepared from it(3). Ac globulin, which is unstable in human blood is stable in bovine blood(4). Accordingly, it was hoped that the AHF of bovine plasma might show a stability corresponding to that of the bovine Ac globulin. This has proved to be the case.

Methods and materials. Collection of blood. All blood specimens were collected in untreated glass containers. One part of 0.1 molar potassium oxalate to 9 parts of blood served as the anticoagulant. Beef, hog, and sheep blood were obtained from freshly slaughtered animals. The plasma was separated within several hours of collection, and was stored in deep freeze until ready for use. Unless otherwise indicated, the AHF content of human plasma was tested within one hour of blood collection. *Prothrombin* was determined by the method of Ware and Stragnell(5) with slight modifications in the case of serum specimens as previously described (6). *Thrombin evolution* was determined in recalcified plasma according to the technic of Pitney and Dacie(7). *Assay of AHF* was accomplished *in vitro* by testing the ability of the unknown specimen to induce prothrombin consumption in the blood of severe, established hemophiliacs who had received no blood or plasma therapy for at least 2 months prior to testing. The details of this method have been published by Spaet and associates (6). In brief, 0.1 cc of the appropriately diluted specimens to be tested was added to 2 cc of hemophilic whole blood, and serum prothrombin content of the resulting mixture determined 4 hours after clotting. Serial dilutions of each blood or fraction were used, and AHF activity was estimated according to the concentration required to elicit 50% prothrombin consumption in the abnormal blood. In no case was any prothrombin consumption demonstrable when 0.1 cc of saline was added to the hemophilic blood, nor was the serum prothrombin ever lower than that of the respective plasma. *Cohn's fraction I* of bovine origin was kindly provided by the Cutter Laboratories, Berkeley, Calif. *"Acid globulin"* was precipitated from plasma by the technic of Ratnoff and Conley(8). *"AHF fraction"* was prepared in the following manner: Bovine fraction I was dissolved in isotonic saline so that the volume was equal to that of the original plasma from which it had been derived. This solution was brought to a temperature of 56°C and maintained at this temperature for 5 minutes to precipitate the fibrinogen. The precipitate was removed by centrifugation and the supernatant solution was absorbed with freshly prepared barium sulfate. Eight tenths moles of barium sulfate were used for each 100 cc of solution, and absorption was allowed to proceed for 30 minutes at a temperature of 37°C. The barium sulfate was removed by centrifugation, and Seitz filtration. The supernatant fluid was then diluted with 19 volumes of distilled water. This mixture was acidified to a pH of 5.4 with 1% acetic acid, as determined by testing in a Beckman pH meter, thus precipitating the active fraction. The precipitate was collected by centrifugation. This material is designated "AHF fraction," and will be thus referred to in the subsequent discussion. The precipitate was stored in deep freeze in its unmodified form, as solutions were found to be denatured by freezing. When ready for use AHF fraction was dissolved in isotonic saline buffered with one part in 10 of 1/15th molar phosphate buffer of pH 7.4. In general, solutions of AHF fraction were made up so that they represented the original volume of bovine plasma from which they had been derived. *Electrophoretic patterns* of plasmas and their derivatives were obtained on filter paper by a modification of the method described by Kunkel and Tiselius(9).

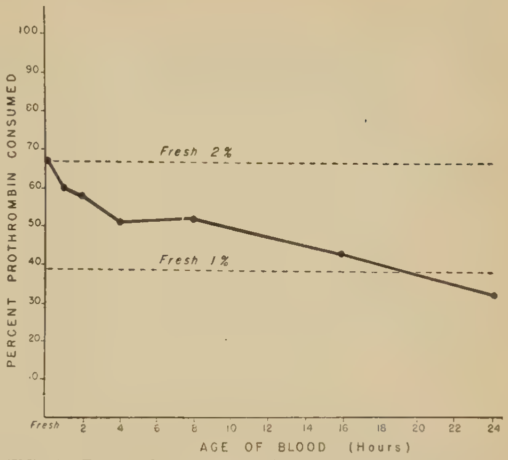

FIG. 1. Loss of AHF activity in human plasma at room temperature. A 2% concentration of plasma was added to hemophilic blood.

Results. Stability studies. Normal human blood was collected from a single donor at intervals as indicated in Fig. 1. The blood samples were each divided into 2 aliquots, one of which was stored at 20°C and the other at 4°C. These blood samples, representing various periods of storage, were simultaneously tested for their ability to restore prothrombin consumption to the blood of a known hemophiliac, which had been collected in a single syringe. Fig. 1 shows the deterioration of AHF activity in stored human blood. It can be seen that there was loss of more than half the activity in 24 hours, and that significant changes were to be found within 4 hours. In the case of this donor, the refrigerated specimens showed essentially an identical pattern of AHF loss, although other investigators have reported improved stability in the cold(10). In contrast, beef, hog, and sheep plasma showed no loss of AHF activity after 24 hours of storage at either 20°C or 4°C. Table I shows the AHF con-

TABLE I. AHF Activity of Human and Animal Plasmas in Hemophilic Blood.

Plasma	Conc. producing 50% prothrombin consumption (%)	Protein content (g/100 cc)
Fresh human	1	6.8
Bovine	.5	6.2
Sheep	.4	5.8
Hog	.2	6.0

TABLE II. AHF Activity of Plasma Fractions in Hemophilic Blood.

Fraction	Conc. producing 50% prothrombin consumption (%)	Protein content (g/100 cc)
Human fraction I (Cutter)	10	.4
" " " (Squibb)	7	.4
" " " (Mich. State Health Dept.)	8	.4
Human acid globulin	2	.8
Bovine " "	1	.9
" fraction I	1	.4
" AHF fraction	1	.1

tent in the various types of plasma studied. None of the human or animal sera had any appreciable AHF activity.

Fractionation. Table II shows the AHF activity of different plasma fractions. Three lots of human fraction I were assayed, each having been produced by a different manufacturer. None of these had more than 15% of the activity of fresh plasma, when considered on the basis of reconstitution to the original volume of plasma represented. On the other hand, bovine fraction I had about half the activity of the original plasma. A similar trend was seen in the acid globulin fractions of the various species tested. Preparations of beef, hog, and sheep plasmas had significantly more activity than that of human origin. The yield of AHF activity in the acid globulin derived from bovine fraction I (AHF fraction) was almost quantitative, despite a considerable reduction in the protein content of this preparation. A further check on the thromboplastic activity of this preparation was accomplished by means of the thrombin evolution test. Small amounts of beef AHF fraction were added to hemophilic plasma, and improvement of thrombin evolution following recalcification was compared to that produced by fresh human plasma. Reconstituted to the original volume of plasma from which it had been derived, the

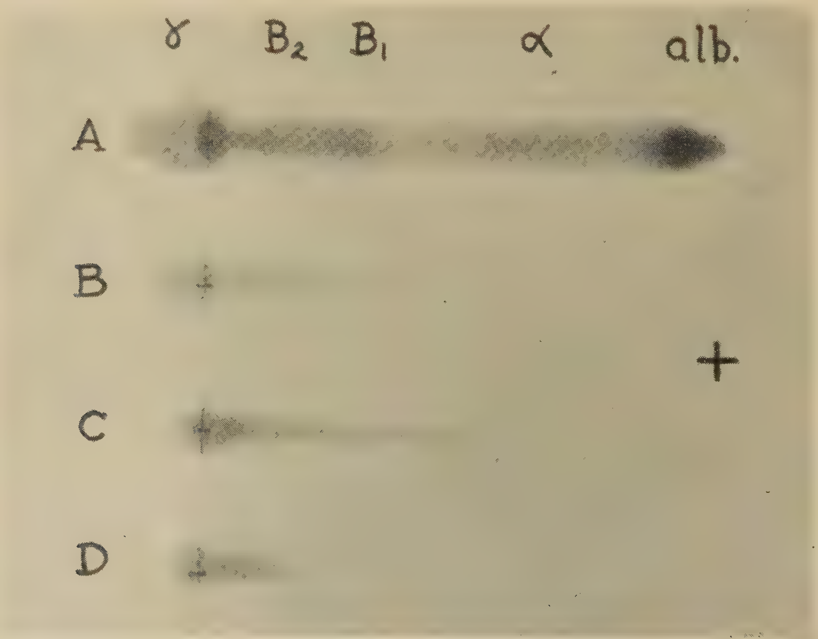

FIG. 2. Paper electrophoretic patterns of bovine plasma fractions. (A) whole plasma; (B) heated fraction I; (C) heated acid globulin; (D) AHF fraction (concentrated 5-fold).

beef AHF fraction was as active as whole human plasma. This finding indicates that the improved prothrombin consumption induced by beef AHF fraction is a true improvement of clotting, and not a nonspecific action resulting in loss of prothrombin. Fig. 2 shows the paper electrophoretic patterns of the various bovine fractions. Both fraction I and acid globulin were mixtures of α_2 and β globulins. AHF fraction was virtually pure β_2 globulin. It is evident that AHF fraction represents a considerable gain in both AHF activity per mg of protein, and purity as compared to previous preparations.

Beef AHF fraction contained no demonstrable fibrinogen, as tested by the addition of purified thrombin (Parke Davis). Neither was there any demonstrable prothrombin, or proconvertin (method of Owren and Aas)(11). Traces of labile factor activity were found, tested by the method of Quick(12). PTC activity(13) was kindly assayed by Dr. Paul M. Aggeler. Although beef AHF fraction was able to shorten the clotting time of *PTC*-deficient blood, 25 fold concentrations did not elicit any improvement in prothrombin consumption. In the precipitated form, beef AHF fraction was stable for at least 3 months in deep freeze; but freezing solutions of the material caused precipitation of the protein with complete loss of AHF activity.

Summary. The anti-hemophilic factor (AHF) of human plasma is unstable to storage, and shows great loss with attempts at fractionation. Bovine AHF is stable to storage, and protein fractions can be obtained which show a good yield of AHF. Beef "AHF fraction" was prepared as an almost pure β_2 globulin, and had about 70 times the AHF activity of fresh human plasma per mg of protein.

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Received September 14, 1953. P.S.E.B.M., 1953, v84.

Particle Size of Soluble Antigen of Rabies Virus. (20632)

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A study of the size and the antigenicity of the complement fixing "soluble antigens" of viruses may throw considerable light on virus structure. Recently Hoyle, Reed and Astbury (1) have shown that influenza virus can be broken down by ether treatment into complement-fixing and haemagglutinating particles. They obtained pure preparations from allantoic fluid and by electronmicroscopy estimated the particle diameter to be 12 mμ.

We have obtained the soluble antigen of rabies virus from infected suckling mouse brains. The particle size could not be determined in the Spinco preparative ultracentrifuge using the angle head (Polson and Linder (2)) because the amount of sedimentation during 2 hours at 30000 rpm was insufficient. We therefore used a technic of diffusion coupled with biological assay.

Method. For determination of the diffusion constants of pure substances which can be obtained in relatively large amounts the conventional optical method of Lamm is most suitable. When substances are difficult to purify a more satisfactory method is to investigate their diffusion constants by quantitative evaluation of their biological activities. A very useful diffusion technic for such measurements is that described by Polson(3,4). The diffusion apparatus consists of 6 multichambered diffusion cells clamped to a base provided with levelling screws. Sharp interfaces between medium and solution were formed by filling adjacent cells with medium and solution and rotating the upper section of the cells until the liquid in the segment above came into apposition with that in the segment below. This was done by watching the index lines on the proximal and distal sides of the cells. The diffusion experiments were conducted in a water thermostat kept at 20°C. After suitable intervals the material that diffused past the initial boundary was isolated from that in the distal portion by rotating the upper sections to their cut-off positions. The activities of the diffusates and original solution were determined by their appropriate methods.

The results of activity determinations of the diffusates are presented graphically by plotting the ratios of concentration in the diffusates C to that in the original solution C_0 against the square root of t, the time in hours. It is a necessary requirement from the laws of diffusion that C/C_0 should be a linear function of $\sqrt{t}$.

The diffusion constants were calculated from these data using the equation

$$D = \left(\frac{C}{C_0}\right)^2 \frac{H^2\pi}{t} \qquad 1$$

Where H is the height of the column of liquid above the original boundary, regarding the chambers in the diffusion cells as cylinders. The value of H in the present experiments has been 3 cm. Estimation of C_0: Since C_0 enters into equation 1 as the second power it is of the utmost importance to have a value for C_0 that differs as little as possible from the real value. To obtain a value for the activity that proximates the true figure, activity estimations were made on the original solution diluted initially to different degrees. The activities thus determined are then multiplied by the dilution factor and the average value of the different determinations then represents C_0.

Experimental. Material. The Flury strain of rabies was adapted to 3- to 4-day-old suckling mice and carried through 20 generations. The mouse infectivity titre LD_{50} of a 10% emulsion in rabbit serum saline of different batches of infected brain material varied between 9 and 10 if the titrations were made on freshly harvested material. The complement fixing titre varied between 1/512 and 1/1024. For diffusion measurements 15 infected baby mouse brains were emulsified in 30 cc saline containing 10% inactivated rabbit serum and spun for 60 minutes at 2500 rpm in a refrigerated centrifuge. The supernatant fluid was dialysed against a large volume of M/15 phosphate buffer pH 8.2 for 48 hours in the cold. The brain extract was next spun for 60 minutes at 30000 rpm in the No.

TABLE I. C.F. Titres of the Diffusates Obtained from the Different Diffusion Cells.

Cell	Time of diffusion in hr	C.F. titre	Avg
A	1	1/2	1/2
B	13	1/4–1/8	1/6
C	20	1/8–1/16	1/12
D	36	1/16	1/16
E	61	1/16–1/32	1/24
F	84	1/16–1/32	1/24

TABLE II. Results of Titrations of Varying Dilutions of the Original Solution.

Dilution	Titre	Titre/Dilution
1	1/384	1/384
1.33	1/256	1/341
1.50	"	1/384
3	1/96	1/288
5	"	1/480
5	"	"
6	"	1/576
6	"	1/384

Avg 1/411 ∴ $C_0 = 411$

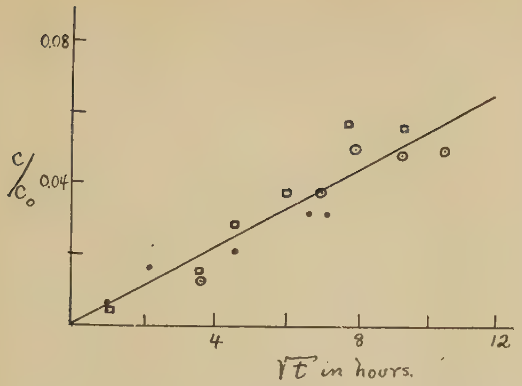

FIG. 1. Ratios of concentration of soluble antigen in diffusates to those in the original solution plotted against the square root of diffusion time in hours.

40 rotor of the Spinco preparative ultracentrifuge to remove all infective virus particles as well as any free lipoid or suspended brain material. Experiments with such extracts spun at different rotor velocities showed that after removal of the infective virus particles there was little further reduction in C.F. titre. The virus free supernatant fluid and the buffer were deaerated in a vacuum before placed in the diffusion cells.

Results. The results of one of the diffusion experiments are given in Tables I and II. These are titrations of the diffusates after different periods of diffusion and titrations of the original material diluted to different degrees respectively.

In Fig. 1 the ratios C/C_0 from three different experiments are plotted against $\sqrt{t}$ where t is the time of diffusion in hours. It is quite evident that C/C_0 is a linear function of $\sqrt{t}$ and that the straight line so obtained passes through the origin. The diffusion constant calculated from this line is 2.7×10^{-7} cm^2/sec.

Particle size of the C.F. antigen of rabies. Particle size calculations from the well known equation of Einstein

$$D = \frac{RT}{N} \cdot \frac{1}{6\pi r \eta} \tag{2}$$

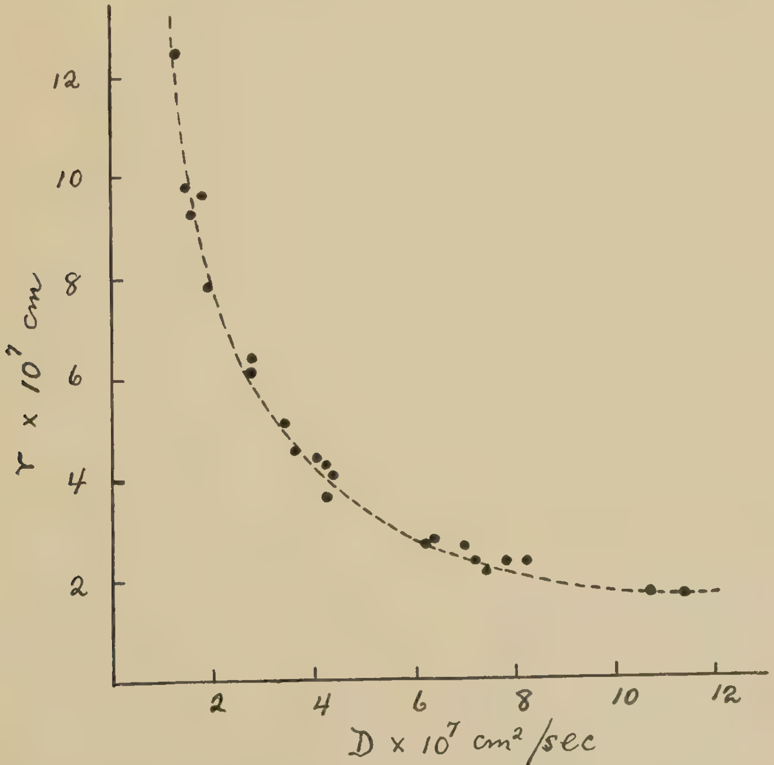

FIG. 2. Curve showing the relationship between diffusion constant and particle radius as calculated from the molecular weights of the globular proteins.

where R is the gas constant, T the absolute temperature, N Avogadro's number, r the radius of the particle and η the viscosity of the medium. This gives the diameter of the hydrated particle which will be appreciably higher than the unhydrated particle. To bring the size, as calculated from diffusion measurements, in line with that measured from electronmicrographs of the C.F. antigen of influenza the diffusion constant of the rabies C.F. antigen was interpolated into a curve relating the diffusion constants with particle radii. These are calculated from the molecular weights of the different proteins in their dehydrated states (Polson)(5). This curve is shown in Fig. 2. When the diffusion constant of the C.F. antigen of rabies is interpolated into this curve a particle radius of 6.2×10^{-7} cm is obtained. This gives a value for the diameter of the particle of 12.4 mμ.

Ultracentrifugation of the rabies soluble antigen. From ultracentrifugation experiments in which the swinging bucket type of rotor of the Spinco ultracentrifuge was used a sedimentation constant of approximately 25 Svedberg units was found. This value corresponded very well with that calculated for a protein particle of 12 mμ diameter. These centrifugation experiments, however, cannot be regarded as final as it was difficult to maintain a constant rotor temperature at velocities $>$30,000 rpm, and the calculations of the sedimentation constant had to be made on runs at lower speeds where only small boundary displacements took place, but during which the rotor temperature remained constant at 7°C.

Summary. 1. A particle size of 12 mμ has been determined by a method of diffusion coupled with biological assay for the soluble antigen of rabies extracted from infected suckling mouse brains. This value agrees with that calculated from the approximate sedimentation constant calculated from the sedimentation rate in the swinging bucket rotor of the Spinco ultracentrifuge. 2. No conclusions regarding the homogeneity of the soluble antigen can be drawn at this stage.

We would like to thank Professor M. van den Ende and Dr. R. Turner for their keen interest in this work.

This investigation was assisted by grants from the South African Council for Scientific and Industrial Research and the Nkana-Kitwe and Chingola Poliomyelitis Research Funds.

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Received September 14, 1953. P.S.E.B.M., 1953, v84.

Survival of Dogs with Bilateral Phrenectomy and Extensive Transection of Intercostal Nerves. (20633)

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According to Brown and Satinsky(1) dogs invariably die after destruction of both phrenic nerves. Partly on this basis they claimed functional restoration of the diaphragm by uniting the vagus in the thorax with the distal end of a severed phrenic nerve. After a suitable interval following such an anastomosis, dogs tolerated section of the remaining phrenic nerve and fluoroscopic studies seemed to indicate that the vago-phrenic anastomosis was responsible for rhythmic contraction of the diaphragm. On the other hand, there are numerous reports antedating that of Brown and Satinsky claiming a high degree of tolerance to section of both phrenic nerves in man and other mammals, including the dog. Bilateral phrenectomy in man without untoward results has been reported by Greene(2), Angle (3), Campbell(4), and according to Lemon (5) by many others. The effects of destroy-ing both phrenic nerves have been studied in the rabbit, dog, goat, horse and monkey by Hare and Martin(6); in the dog by Lemon (7), Jappeli(8), and Feldman and Morrison (9). All of these authors agree that animals so treated survive the procedure without marked respiratory distress, provided they are not very young. Hare and Martin found that young rabbits die when both phrenic nerves are cut, and similar results were reported for kittens by Coombs and Pike(10). However, Coombs and Pike also found that mature cats tolerate the procedure very well. According to Lemon(7) not only will dogs tolerate bilateral phrenectomy but section of most of the intercostal nerves in addition.

Although the earlier reports seem convincing, the experience of Brown and Satinsky led us to do a series of bilateral phrenectomies in dogs. Since it was soon apparent that dogs do survive section of both phrenic nerves, the work was extended to determine the effects of still further reduction in respiratory musculature.

Methods. Eight grown but young dogs were subjected to section of both phrenic nerves under nembutal anesthesia and with aseptic precautions. In 6 animals the phrenic nerves were severed in the neck, and in 2 these nerves were cut 1 cm above the diaphragm. After some days or weeks several of the dogs were subjected to further reduction in the number of active respiratory muscles by cutting intercostal nerves, and in one instance by removal of possible accessory muscles and spinal cord transection. When finally sacrificed, the chest was opened in each case and the phrenic nerve remnants were strongly stimulated with induction shocks. Following stimulation, a portion of each nerve was taken distal to the point of original section and placed in 1% osmic acid. The osmicated pieces of nerve were examined microscopically for any signs of intact fibers in carefully teased preparations.

Results. All dogs, except the first which was lost during the operation, survived bilateral phrenectomy without difficulty. Immediately after section of the second phrenic nerve, each dog exhibited a pronounced increase in costal movements. This increase became progressively less noticeable during the first week and did not change appreciably thereafter.

The first dog to be sacrificed, Dog 2, had lived uneventfully for 5 weeks following cutting the phrenic nerves. The animal was anesthetized and then subjected to the following in succession: transection of the spinal cord between the 10th and 11th thoracic arches, ligature of the axillary vessels and section of the roots of the brachial plexus, section of the long thoracic nerve, transection of the scalene and infrahyoid muscles, cutting of all but the upper 2 intercostal nerves near their origins on the right side, cutting all but the upper 4 intercostal nerves on the left side. While working on the left fourth intercostal nerve the pleura was punctured and the dog expired after a few gasping movements. Until the pleura was inadvertently opened this animal was breathing heavily with the remaining intercostal muscles but showing no signs of respiratory distress. Since this experiment indicated an enormous reserve of respiratory musculature in phrenectomized dogs, the other animals were subjected to different types of reduction in their intercostal musculature and permitted to live. It was established that a phrenectomized dog will live after either the upper or the lower 6 intercostal nerves have been severed bilaterally, when all intercostal nerves are cut along the mid-axillary line, and when only the 3 upper pairs remain. One dog lived for 4 hours with only the lower 6 intercostal nerves of one side intact.

Complete phrenic section was confirmed in all animals by faradization of the distal stumps and examination of teased osmic acid-fixed preparations. In no instance did strong stimulation cause any movement of the diaphragm, and no intact fibers were found in any of the material prepared for microscopic study.

Discussion. After finding that dogs with both phrenic nerves cut in the neck survive in excellent shape, it seemed possible that doing this operation by an intrathoracic route might in some way result in a different outcome. However, such is not the case, so the authors are left without an explanation of the recent report that dogs will not survive bilateral phrenectomy. On the contrary, it seems that barring accidents incident to surgery this animal will always or nearly always survive. This conclusion is buttressed by the fact that half and even more of the intercostal nerves can be destroyed in addition without causing death.

Summary. Seven dogs were kept in good health and without respiratory distress for periods varying between 9 and 67 days after bilateral phrenectomy. All of the animals were eventually sacrificed, none died as a result of paralyzing the diaphragm. Electrical stimulation and microscopic examination proved that the phrenectomies were complete in all cases. It was established that following paralysis of the diaphragm at least half of the intercostal nerves, either upper or lower, may be sectioned without loss of the animal. The conclusion drawn is that reports or statements to the effect that the diaphragm is essential for life in otherwise intact grown dogs are erroneous.

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Received September 14, 1953. P.S.E.B.M., 1953, v84.

Effect of Insulin on Maximal Rate of Renal Tubular Uptake of Glucose in Non-Diabetic Humans.* (20634)

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Although the various physiological effects of insulin are well known, the specific mode of action of the hormone has not been clearly delineated. At present, it is generally believed that insulin facilitates, in some manner, the phosphorylation of glucose which introduces carbohydrate into the metabolic processes of the cell. The exchange of glucose between glomerular filtrate and renal tubular cell appears to be a unidirectional process and is characterized by a maximal rate of tubular cell uptake of glucose. Glucose is completely removed from the filtrate until filtered load of glucose is increased to a point where the tubular resorptive mechanism is saturated. Further increments in filtered glucose appear in the urine. This situation would appear to provide a unique opportunity to study the effect of insulin on the rate of cellular uptake of glucose in the intact animal as contrasted to most *in vivo* work which has been concerned with the net exchange of glucose between cell and perfusing medium.

Methods. Twelve male subjects aged 47-68 years who were free of evidence of renal disease, cardiovascular disease, and derangement of carbohydrate metabolism were studied in the fasting state. The general procedure outlined by Goldring and Chasis(1) for the determination of Tm glucose was followed. After suitable priming dose, glucose was infused as a 30% solution in water at a rate of approximately 1.5 g per minute throughout the entire procedure. Inulin was added to the infusion fluid in quantities sufficient to maintain plasma levels of 20-25 mg %. Blood was sampled at the mid-point of each urine collection period through an in-

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* The author is indebted to Elsie Beard for technical assistance and to Dr. Milton Landowne for suggesting an hypothesis to explain the observed reduction in Tm.

TABLE I. Effect of Insulin on Tm Glucose,* mg/min.

Subject	Age, yr	Height, cm	Wt, kg	Control period 1	2	3	Post-insulin period 4	5	6	7	8
L.	64	172	61.7	292	264	255	266	240	220	272	252
S.	48	170	59.9	288	254	282	298	321	261	321	299
B.	47	172	68.5	324	338	401	339	306	272	287	286
D.	65	162	61.0	158	133	126	149	134	116	146	127
B.	68	172	85.6	254	261	243	255	261	189	231	310
McK.	63	172	86.0	227	225	234	210	186	176	174	204
H.	52	172	54.1	217	218	212	215	194	187	232	210
J.	62	178	70.0	289	242	276	278	247	257	270	341
B.	65	165	61.0	341	322	352	390	347	286	306	299
A.H.	47	176	65.1	376	379	377	360	302	350	333	382
C.	65	168	56.8	243	236	256	206	154	217	198	217
F.C.	63	172	60.4	328	340	334	339	334	284	338	347
Mean				278.1	267.7	279.0	275.4	252.2	234.6	259.0	272.8
σ				58.5	64.5	73.7	69.6	69.2	60.1	60.6	69.8
σ_{MD}				17.6	19.4	22.2	21.0	20.9	18.1	18.3	21.0

Glucose reabsorption ($\mathrm{Tm_G}$) is expressed in mg/min. and is derived as the difference between the rate of filtration of glucose and the rate of urinary excretion of glucose.

* Periods 1-3 represent 3 consecutive control urine collection periods, each of 12 min. duration. Periods 4-8 represent 5 consecutive 12 min. urine collection periods following the intravenous inj. of 20 units crystalline zinc insulin.

Values are not corrected for body surface area.

dwelling needle in the femoral artery. Heparinized blood samples were centrifuged, and plasma was removed within 20 minutes after the sample was obtained. Urine collection periods were terminated by washing out the bladder with physiological saline. Following 3 control urine collection periods, 20 units of crystalline zinc insulin were injected intravenously and 5 successive urine collection periods, each of 12 minutes duration were obtained over the 60-minute period following the injection of insulin. Inulin was determined in diluted urine and in plasma filtrate by the method of Harrison(2). Glucose was determined in diluted urine and plasma filtrate by the spectrophotometric method of Nelson(3).

Results. The intravenous administration of insulin in the present series converted the rising plasma glucose level of the control periods to a progressively decreasing level in the face of continuation of the infusion of glucose at the same or at somewhat augmented rates (Table II). Despite this increase in overall net cellular uptake of glucose, the rate of glucose uptake by the renal tubular cells from the tubular lumen actually decreased (Table I). The decrease in Tm glucose, although small, occurred in all subjects during period 6 and was statistically significant at the 5% level of confidence for period 5 and at the 1% level of confidence for period 6.† That the decrease in $\mathrm{Tm_G}$ was not due to a decrease in the filtered load of glucose can be seen in Table II. The rate of filtration of glucose was as great or greater following the injection of insulin than during the control periods. The ratio of filtered glucose to reabsorbed glucose was consistently greater during the post-injection periods than during the control periods. This increase in L/T ratio was due to both a slight increase in the glomerular filtration rate and a slight decrease in the rate of tubular uptake of glucose.

Discussion. The slight, but statistically significant, decrease in $\mathrm{Tm_G}$ produced by the administration of insulin to non-diabetic human subjects in the present study is in accord with the findings of Farber *et al.*(4) who reported a reduction in $\mathrm{Tm_G}$ when insulin was administered to diabetic subjects. The insulin induced depression in $\mathrm{Tm_G}$ is clearly not a phenomenon peculiar to the diabetic individual, as has been suggested, but one which occurs in the non-diabetic subject as well.

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† Since each subject served as his own control, tests of significance were based on the differences between the mean of 3 control periods and the period tested computed individually for each subject.

TABLE II. Glomerular Filtration Rate, Plasma Glucose Level, and Rate of Glucose Excretion Following Insulin.*

		Control period			Post-insulin period				
		1	2	3	4	5	6	7	8
Plasma glucose, mg/100 cc	Mn	500.5	545.4	578.3	606.5	599.1	567.8	550.0	522.7
	σ_{Mn}	16.9	15.3	17.4	17.6	19.9	23.5	26.1	32.1
GFR, cc/min.	Mn	95.1	93.3	95.4	97.6	95.6	94.7	99.3	102.5
	σ_{Mn}	5.0	5.6	6.3	6.0	6.3	6.7	6.6	7.3
Filtered glucose, mg/min.	Mn	476.7	509.5	550.1	588.7	571.2	531.0	538.9	538.1
	σ_{Mn}	30.5	33.9	37.2	40.5	41.3	36.4	35.9	41.9
Glucose excretion, mg/min.	Mn	198.5	242.0	271.0	312.4	319.1	296.4	279.9	265.2
	σ_{Mn}	23.3	26.6	26.4	30.2	29.1	26.5	25.2	26.9
Load/T	Mn	1.74	1.99	2.02	2.19	2.34	2.33	2.13	2.01
	σ_{Mn}	.09	.11	.10	.12	.12	.13	.11	.11

Mean values for entire group of 12 subjects.
* See footnote Table I.

In an attempt to reconcile the somewhat paradoxical findings wherein the rate of renal tubular reabsorption of glucose actually decreases during the period when insulin produces a marked increase in overall net cellular uptake of glucose, it will be well to recall that the dynamics of tubular uptake of glucose from the tubular lumen differs in several respects from that of glucose uptake by other cells such as those of skeletal muscle. Glucose is completely reabsorbed by the renal tubule from the filtrate until filtered load of glucose is increased to a point where the reabsorptive mechanism is saturated. The limitation in rate of uptake of glucose from the tubular lumen is most likely due to a limitation in the rate of regeneration of some intracellular substance(5), presumably ATP. On the other hand, glucose is removed from extracellular fluid by muscle at a relatively slower rate. ATP is not a limiting factor in the phosphorylation of glucose by muscle tissue(6), and the rate of cellular uptake is enhanced by insulin. The reabsorption of glucose from the tubular lumen may be considered as a reaction in which glucokinase is abundant relative to the concentration of one of the reactants, ATP; while the cellular uptake of glucose by muscle may be viewed as a reaction in which the enzyme exists in relatively low concentration. Under these circumstances, if the point of action of insulin is on the hexokinase reaction, the hormone would be expected to exert no direct effect on the rate of glucose uptake from the tubular lumen by the tubular cell, and to increase the rate of uptake by muscle cell. The observed reduction in glucose Tm after administration of insulin would not be explained by the above considerations. However, if there exists in the renal tubular cell a mechanism for glucose uptake from peritubular fluid similar to that which exists in muscle tissue, augmentation of this rate of glucose uptake from the blood as a consequence of insulin administration could conceivably reduce the rate of glucose uptake from the tubular lumen by decreasing the amount of available ATP.

Summary. 1. The maximal rate of renal tubular reabsorption of glucose was determined before and after the intravenous administration of 20 units crystalline zinc insulin in a series of 12 non-diabetic male subjects who were free of evidence of cardiovascular or renal disease. Following the administration of insulin, there occurred a slight, but statistically significant, decrease in Tm_G during the period extending from 12 to 48 minutes after injection. 2. The significance of these findings, with respect to the fundamental action of insulin, is discussed.

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Received September 15, 1953. P.S.E.B.M., 1953, v84.

Growth and Mortality Effects Produced in the Early Chick Embryo By Antiserum. (20635)

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The how of ontogeny or epigenesis in the earlier stages of embryonic development is still far from being answered. It is known that at the stage where competency begins organ materials are laid down in a strict and orderly fashion. A series of experiments was designed to investigate the pre-differentiation stages in the chick embryo. A variety of agents was used in an effort to modify the growth pattern of early embryos. The most successful of these agents was found to be whole embryo anti-chick serum produced in hamsters. There are no published reports in the literature which deal with the effect of whole embryo antiserum on the chick embryo. A few workers have reported experiments of this sort on the amphibian embryo(1-3). Previous experiments were done under a variety of conditions and the results are comparable. They may be summarized by saying that the amphibian experiments showed total or partial destruction to the embryos, retardation of growth, production of anomalies and an increased mortality rate in the embryo. The experiments in the present report gave results similar to those in the amphibians. They produced such clearly defined effects that certain new conclusions may be reached. By injection of antigenic embryo materials of specific time intervals, embryonic development in recipient embryos is limited to the stage of the embryo which was used as antigen.

Methods. Fertile eggs, fresh from the yard, were incubated in groups of 100 for 24 hours, 72 hours and 6 days. The eggs were opened with a motor disk and each living embryo removed with a minimum of yolk material and none of the membranes. Technical difficulties are present at the 24-hour stage, the fragility of the embryo at this time necessitates incision of the vitelline membrane and gentle suction removal of the embryo with a sharp, large bore needle and wet tuberculin syringe. The embryo materials after removal were placed in separate lots and thoroughly emulsified in normal saline by syringe suction and expulsion or grinding in a mortar without abrasive. Embryo material of 24 hours, 72 hours, and 6 days was injected intramuscularly into 3 separate groups of hamsters. The injections were started with .5 ml of a rather opaque solution, in which there were particles, in the first injection and increased up to 2.5 ml in the 13th injection. The injections were given every third day and after the last injection a period of 2 weeks was allowed to elapse before the hamster was bled. Those animals which survived the first intracardiac bleeding were rebled on the 21st day after the last injection. The hamster anti-chick embryo serum was tested for sterility and only those lots which were sterile were used in the final experiments. Anti-chick embryo antiserum was not treated with preservative nor were absorption or complement fixation or precipitin tests performed upon the serum. Fresh fertile but unincubated eggs were opened and a window made over the embryo site. In each series approximately .01 ml of anti-chick antiserum was dropped upon the vitelline membrane directly over the embryo site or .01 ml was injected with an 1½ inch, size 26 needle, beneath the membrane into or near the embryo site. There were no differences in the results of these two methods of administration except that a higher mortality resulted in the injected embryos. The same finding of heightened mortality was found amongst the controls where normal hamster serum was injected into the embryo site. We expect this was due to direct damage to the embryo, rather than any antibody effect. Adequate numbers of controls, using normal saline as drop and injection material and normal hamster serum were run. The treated eggs were examined every 24 hours throughout the time of their survival and observations recorded.

Results. 24 hours anti-chick embryo antiserum series. In these eggs embryonic development was markedly retarded and usually

TABLE I. Effect of Hamster Anti-Chick Embryo Serum on the Chick Embryo.

Treatment	No. embryos treated	Developmental stage				Abnormalities	Mortality
		16 Blastula gastrula	24 Prim. streak blood islets	72 30-35 somites	6 days		
24 hr	129	25	47	12	2	Cystic growths	43
72 "	168	3	19	41	5	Monsters	100
6 day	60	4	4	5	29	Small retarded embryos	18
Controls	142	0	2	0	0	Normal	7

stopped at the 24-hour stage with death of the embryo at this stage. Occasionally early blood islet formation was found but the embryo rarely progressed beyond this stage. Malformed gastrulae and bizarre-shaped protoplasmic masses appeared. They did not grow well and all attempts to transplant them upon more advanced membranes failed. Rarely an embryo reached the primitive streak stage before death occurred (Table I).

72-hour anti-chick embryo antiserum series: This group developed to more advanced stages than the 24-hour group. There was definite tendency to micro-embryos, some of them reached the heart-beat stage. Malformations of various body parts became more pronounced. Most of these were in the cardiac system with different kinds of vascular and cardiac anomalies, or in the cranial end of the embryo with different kinds of heads and eyes much as micro or macrocephaly, unusually shaped heads and eyes. Occasional 2 or 3 tailed embryos or excessive numbers of limbs were noted. The great majority of embryos were dead by the 72-hour stage: a few survived until the fourth day (Table I).

6-day anti-chick embryo antiserum. Embryos in this group showed some malformations but their number and severity were less than in the previous groups. Some embryos continued to grow until hatching but there was a mortality rate in the group more than twice that of the control series (Table I).

Discussion. Anti-chick embryo antiserum contains a factor which placed in proximity to the early developing embryo produces stoppage of growth in stepwise fashion, anomalies and heightened embryo mortality. The interest here is not in the serological aspect but in the effect upon the embryo's growth pattern. The same kind of experiments done on developing newt embryo explants gave results which caused Clayton(2) to suggest that either an antigen or antigens appear during gastrulation, also the possibility that different parts of the embryo contain common as well as specific antigens. The present report seems to indicate even more clearly that, as the embryo develops new antigens make their appearance. It is certain that the 24- and 72-hour chick embryos contain substances which are antigenic and their antibodies cause development to be reduced and mortality rate to be increased. The mechanism for the above is not clear since the experiments are of limited nature. Critical factors such as concentration of serum, antibody titer and kinds of antibody present, also sites of antibody action must be determined in future experiments.

It may be considered that the appearance of new antigenic material, on a time basis, in the chick embryo is related to the development of new protein complexes. In this field an application of the Schechtman type of studies(4) would be profitable. The present work is suggestive of the development of qualitatively different protein complexes in the embryo concurrent with the embryo's development.

Summary. Using the described experimental conditions whole anti-chick embryo hamster serum placed in proximity to the preincubated chick embryo stops the development of these embryos at a time which corresponds to the time the embryo antigen was obtained. Even though the experimental factors are complex the experimental series differed sharply from the controls. The anti-embryo serum also produced anomalies and a high mortality in the treated series. These experiments show clearly that chick embryos contain distinctive antigenic materials which are related to the embryo's age. The instep order of the results

in the recipient embryos points strongly to the development of qualitatively different protein complexes in the embryo concurrent with the embryo's growth.

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Received September 18, 1953. P.S.E.B.M., 1953, v84.

Close-Packed Array of Virus-Like Particles within Cells of a Human Skin Papilloma.* (20636)

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A distinctive type of wart found on the plantar surface and elsewhere on the skin of man has been described previously(1-3). In contrast to the more common wart this papilloma is characterized by certain cytological features including an intranuclear inclusion body and cytoplasmic masses. In addition, virus-like spherical particles measuring 68 μ in diameter were demonstrated in suspensions of ground tissue examined with the electron microscope. This report is concerned with the location and arrangement of these particles in the papilloma cells as seen in thin sections.

Methods. The present report is based upon a study of 3 papillomas. The excised warts were fixed either in osmic acid after the method of Palade(4) or in 10% formalin if the tissue was not available for the former.

Dehydration in alcohol was followed by imbedding in butyl methacrylate with or without added methyl methacrylate (10%) and polymerization by 2-4 dichlorobenzoyl peroxide (2%) by placing in an oven at 60°C for 18 hours. Sections were cut using a glass knife(5) in a Servall cantilever microtome.[†] They were examined without removal of plastic using an RCA electron microscope model EMU 2E. Sections from the same ribbon were mounted on albumin-coated glass slides, soaked in xylol to remove the plastic, and stained with Weigert's iron hematoxylin or methylene blue to aid in orientation by light microscopy. Staining was carried out by leaving the slide in either dye for one to 2 hours and washing in water. It was then dehydrated in alcohol, cleared in xylol and mounted. Ground suspensions of papillomas were prepared for electron microscopy and shadowed by palladium as previously described(1).

Results. Cells of the different epidermal layers constituting the papilloma are distinguishable in thin sections under the electron microscope since they present about the same features as in conventionally stained paraffin sections and hematoxylin-stained methacrylate sections under the light microscope. The cells in the Malpighian layer of this type of papilloma contain a spheroidal intranuclear inclusion body as well as a few irregular cytoplasmic masses.

Particles resembling the virus-like particles obtained from ground suspensions of these warts have been seen in electron micrographs in the nuclei of cells of the Malpighian layer (Fig. 1-3). These particles are small points at low magnification and spherical at sufficiently high magnification, and generally are arranged in regular rows. The nuclear membrane often appears double and may be separated, apparently by artefact, from the mass of virus-like particles within. It has not been observed to contain virus-like particles. The particles are in straight rows sometimes at right angles to one another, while at others

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* Supported by a grant from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

[†] Manufactured by Ivan Sorvall, New York City.

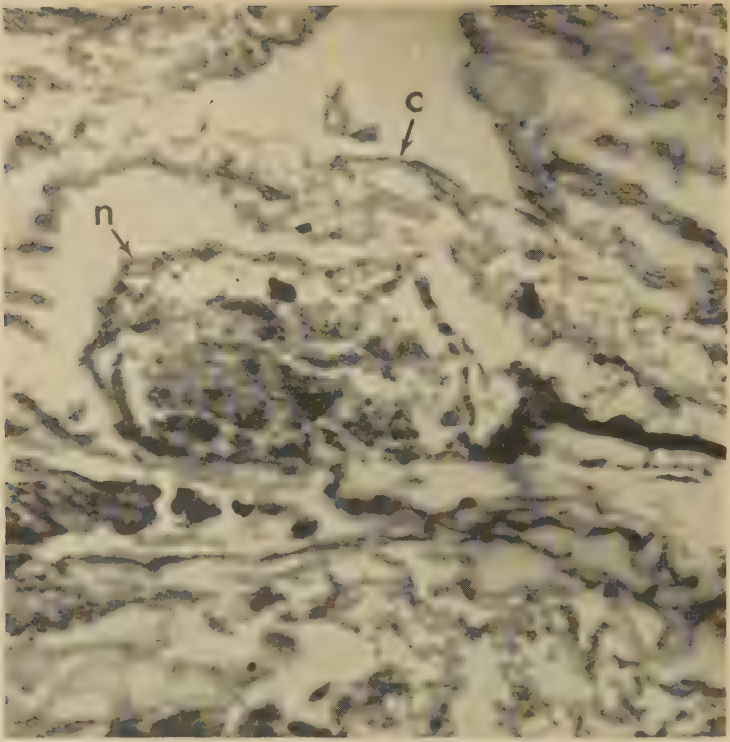

FIG. 1. Nuclear membrane of cell in stratum Malpighii indicated by n, cytoplasmic border by c. Intranuclear inclusion body not present in this particular section through this cell. Gaps in section are due to artefact of preparation, methacrylate not removed. Osmic acid fixation. ×7700.

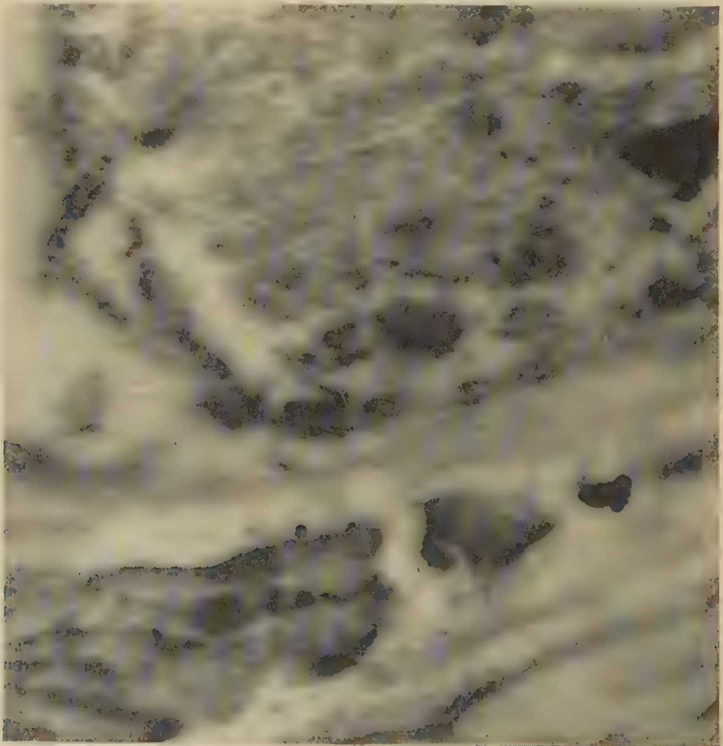

FIG. 2. Further enlargement of negative of Fig. 1. Nuclear material can be seen to be particulate in contrast to the adjacent cytoplasmic substance. Osmic acid fixation. ×21000.

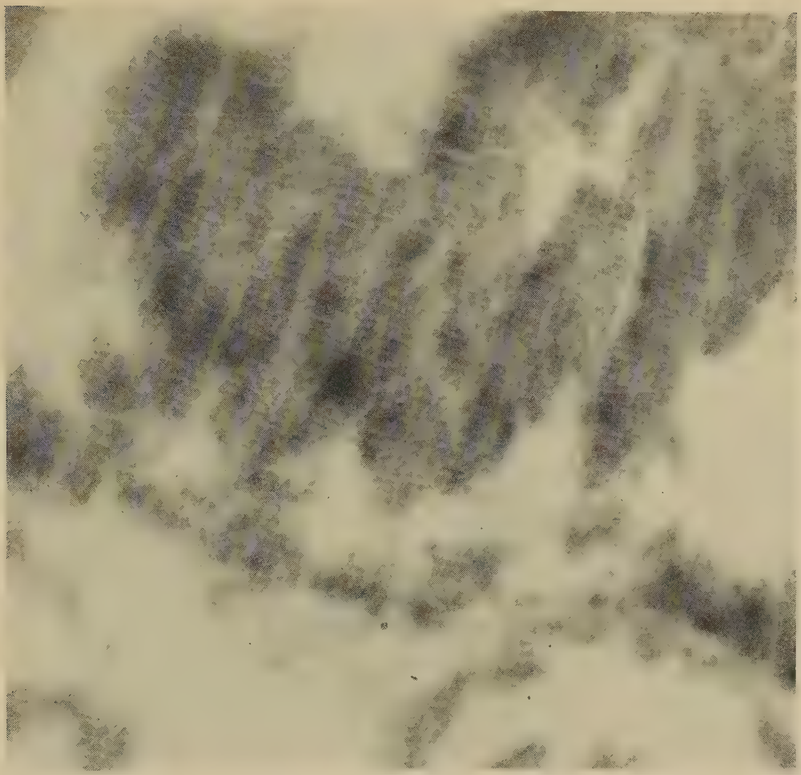

FIG. 3. Small portion of nucleus with nuclear membrane evident and a few adjacent tags of cytoplasm. Gaps are due to artefact of preparation, methacrylate not removed. Nuclear substance composed entirely of particles arranged in rows. Osmic acid fixation. ×33700.

at an angle of approximately 120°. This orientation has been seen to disappear under slight distortion as for example may result from a tear in the section due to heat from the beam, even though this may be at some distance from the cell under observation.

The particles are uniform and spherical (Fig. 6) both in ground suspension of the papillomas and as they lie in the cells. Their diameter measures 56-80 mμ, averaging 68 mμ, in preparations from suspensions when they are separated, and 50-53 mμ averaging 52 mμ when packed(1). In the sections their diameter measures in different preparations from 20-38 mμ. The shrinkage during fixation and dehydration perhaps accounts for the smaller size of the particles in sectioned material. There is, of course, no assurance that the diameter obtained from suspensions is not also distorted somewhat due to dehydration. The surface of the particles is smooth or at times slightly rough or pitted in the shadowed suspension preparations while in sections their outline is uniformly smooth and circular. In preparations from suspensions the particles have not been seen unless shadowed. In sections the particles diffuse electrons more completely than the substance between them. All of the material within the nucleus of the affected cells except for the inclusion body consist of clumps of regularly arranged particles. The intranuclear inclusion body has little discernible internal structure other than an occasional faint irregular granularity. It is sharply demarcated as are also the cytoplasmic masses which have about the same appearance in section. Both of these structures have greater electron scattering power than the rest of the cytoplasm, whether fixed in osmic acid or formalin. In the examples thus far studied virus-like particles have not been recognized in the cytoplasm of the cells of the Malpighian layer although they fill the nuclei of these cells as

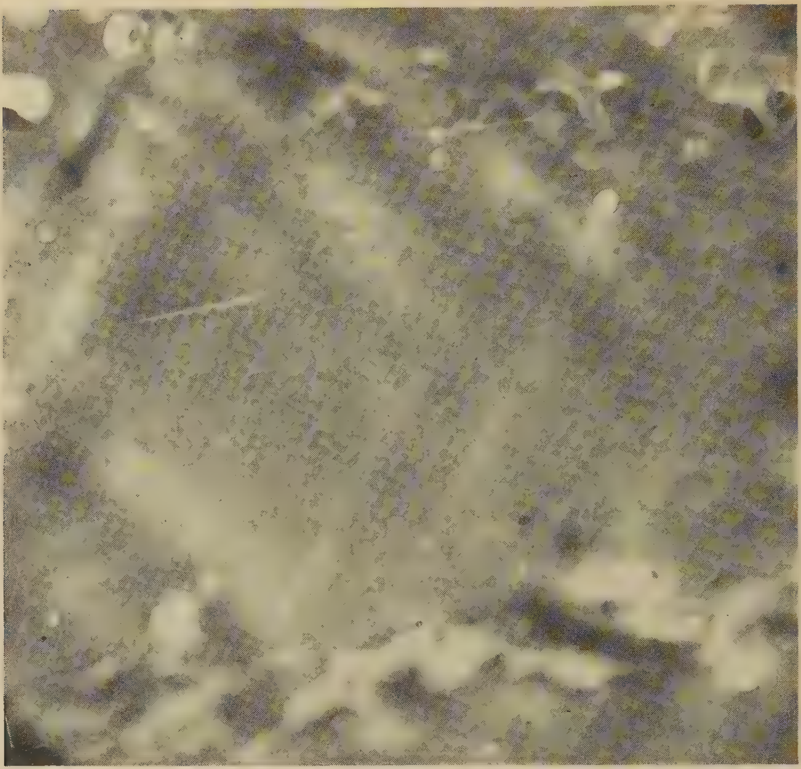

FIG. 4. Cell from lower stratum corneum with interdigitations between adjacent cells evident at its upper and lower border. Nucleus not identifiable in cells of this layer. All of the cytoplasm but the peripheral zone contains regularly arranged particles visible as a meshwork of straight lines. Methacrylate not removed. 10% formalin fixation. ×14500.

described. The cells from the lower stratum corneum are often slightly separated making the interdigitations between them conspicuous. These cells no longer contain nuclei; however, they do contain solid areas of regularly arranged rows of particles as in the nuclei of the cells of the Malpighian layer just described (Fig. 4 and 5). These areas of closely packed particles fill the entire cell except for a peripheral zone just within the cell border which includes the interdigitations and a narrow zone within. The peripheral area appears to be without structure. No particles have been identified in the intercellular space nor in the cells of the uppermost layers of the stratum corneum where the cornified cells are present as irregular partly separated strands.

Discussion. Electron micrographs of thin sections of a distinctive type of human skin papilloma reveal a close-packed array of spherical particles within the nucleus of the cells at one stage of development and in the cytoplasm at a later stage of development. These spherical particles are very easily demonstrated in ground suspensions of the tissue of the papilloma(1). The cells of this type of wart differ from those of the more common wart in that they contain an intranuclear inclusion body (as distinct from the nucleolus) and cytoplasmic masses as well as other features of abnormal cellular development(3).

Virus-like particles have been found only in the nucleus of cells of the Malpighian layer. And, indeed, there is nothing other than masses of virus-like particles identifiable in these nuclei, except the intranuclear inclusion body. The virus-like particles are generally arranged in regular rows. The intranuclear inclusion body presents an almost uniform cut surface and a sharp limiting border with no virus-like particles visible. In cells of the lower stratum corneum, where no

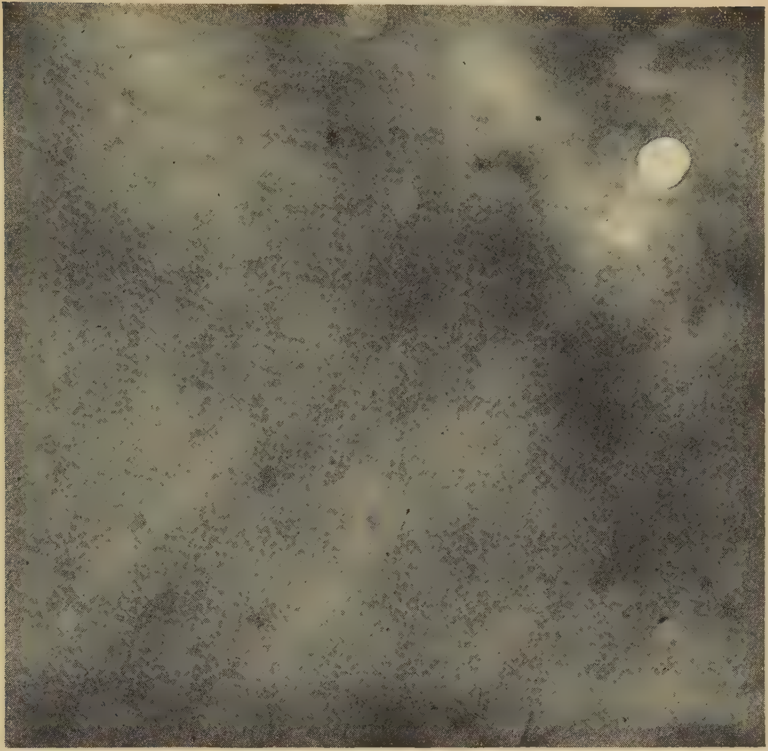

FIG. 5. Same cell as in Fig. 4, higher magnification. Orientation possible from hole in right upper corner. Lines resolvable into particles in close-packed array. 10% formalin fixation. ×30000.

nuclei can be seen the entire cytoplasm, except the outer rim, consists of a solid mass of particles with an arrangement similar to that in the nuclei from the Malpighian stratum. The possibility exists that the virus-like particles here have been derived from the nucleus which as a recognizable structure has disappeared. The appearances suggest, however, that the amount is greater than merely that which had previously filled the nucleus. Further examples of consecutive stages are necessary to determine this as well as to correlate the development of virus-like particles with the cytological and histochemical features already established. There are certain implications, however, inherent in the finding that the particles are generally present only in a close-packed array throughout a large portion of the volume of the cell; such an arrangement could result from the formation of particles *in situ* by a process of crystallization or by their alignment under restricting or confining pressure. Such structure is seen in preparations of macromolecular solids consisting of plant virus proteins which form true crystals(6,7). In preparations of virus-like particles from ground suspensions from these papillomas, the particles being spherical and of uniform size, aggregate in bunches at times quite apparently through the drying forces of the surface film. This is illustrated in Fig. 6, where they have been caught on one side of a collagen fibril. Although here and there a few particles appear to be grouped in a close-packed array the bulk do not. The preparation illustrated in Fig. 1A in the first publication on these papillomas(1) shows a close-packed array with at least 2 layers, suggesting a crystal-like formation. The perfection of structure here resembles that observed in the sections of cells. It is to be interpreted perhaps as an actual portion of a cell which had been incompletely ground and dispersed, although there has not been opportunity to verify this.

It is our belief that the particles actually

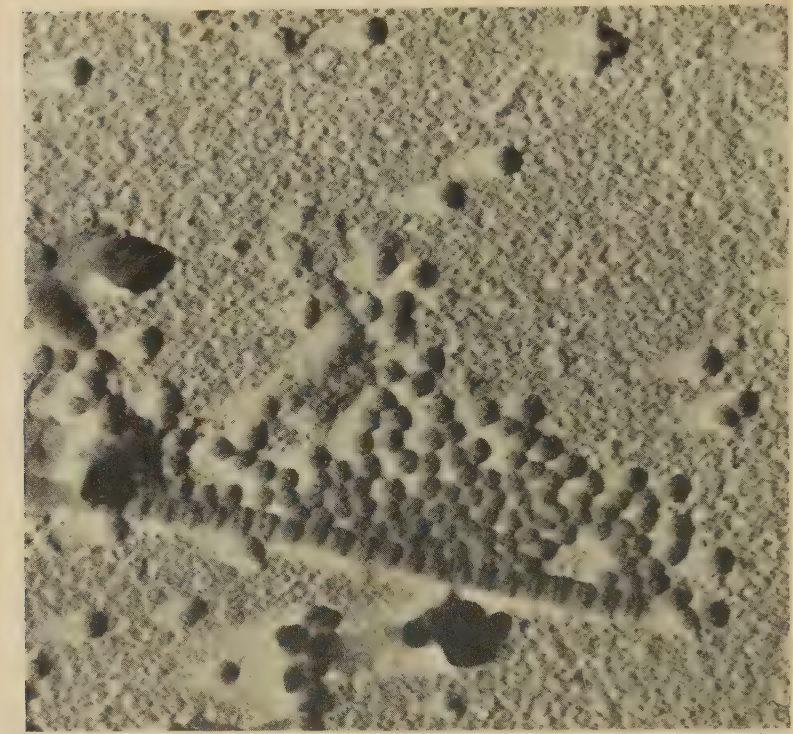

FIG. 6. Preparation of ground suspension of fresh papilloma tissue shown in section in Fig. 3. Shadowed with palladium (angle 1:7). Particles have come to lodge during drying on one side of collagen fibril contaminant from dermis. Loose packing is seen. ×50000.

represent virus particles and are the cause of the type of papilloma described. Inasmuch as this has not yet been established the term virus-like has been used for the particles.

Summary. Intracellular spherical particles have been identified under the electron microscope in thin sections of a particular type of human wart which yields similar particles in ground suspension. In cells of the Malpighian layer of the neoplastic epidermis constituting the papilloma, the particles lie in the nucleus, but have not been recognized in the cytoplasm or in the intranuclear inclusion body or in the discrete cytoplasmic masses characteristic of this type of wart. In cells of the lower stratum corneum of the wart where the nucleus is not recognizable the particles occupy all of the cell except for a narrow peripheral border. The arrangement of the particles is generally regular and like that of close-packed spheres.

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Received September 18, 1953. P.S.E.B.M., 1953, v84.

Accentuation of Dietary Amino Acid Deficiency by Raw Soybean Growth Inhibitor. (20637)

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The present report is an extension of our studies on the inhibition of chick growth by raw soybean meal in the diet, as affected by partial dietary deficiencies of methionine, lysine, arginine, and isoleucine(1). The last 3 examples were shown to be analogous to the well known case of methionine which has long been recognized as the amino acid inadequately provided in whole soybean protein when fed as the only source of protein(2). The general nature of this interaction between the essential amino acid level and the growth inhibitor has now been demonstrated in the additional case of tryptophan.

Methods. The 20% protein chick diet for Exp. 1 was the same as used previously, except that meat scrap provided 15% protein in the diet in addition to the 5% quota of raw soybean meal protein. This amount of raw soybean protein has been shown to impart nearly all of the growth inhibition that may be observed with higher levels of raw soybean meal(1,3). Methionine at a level of 0.3% was also added. Duplicate groups of nine carefully selected day-old New Hampshire chicks were placed on each diet and raised to 28 days age.

The results are given in Table I. In a second experiment we employed the 30% protein diet of Hill, *et al.*(4) who have reported that amino acids would not prevent or reduce any of the growth retardation brought about by feeding raw soybeans to chicks. It was indicated by calculation that the possible deficiencies to be encountered would be limited to methionine and arginine. Only these amino acids were added, instead of the large total amount of some 11 amino acids added to the diet by Hill *et al.* The amino acids replaced an equivalent amount of casein. The results of this experiment are given in Table II.

TABLE I. Effect of Tryptophan Addition to a Raw Soybean Meal and Meat Scrap Diet.

Supplement or treatment of diet	Avg wt of chicks at 28 days, g
None	131.3
L-Tryptophan, 0.2%	254.5
Soybean meal portion heated	354.5
Soybean meal portion heated, plus L-Tryptophan 0.2%	327.3

TABLE II. Effect of Methionine and Arginine Additions to a Raw Soybean, Casein and Fish Meal Diet.

Supplement or treatment of diet	Avg wt of chicks at 28 days, g
None	247.8
DL-Methionine, 0.4% plus L-Arginine, 0.3%	307.6
Soybean meal portion heated	316.5

Discussion. The diet used in Exp. 1 was expected to be slightly deficient or barely adequate in tryptophan content. When the soybean growth inhibitor was destroyed by heating, the chicks grew well and there was no increased growth response from added tryptophan. However, the diet containing the soybean protein in raw meal state permitted only relatively poor growth. The growth was distinctly improved by the addition of tryptophan (Table I). This example may be added to those which show that the increased growth inhibition, caused by interaction of the raw soybean inhibitor with a partial deficiency or a barely adequate level of an essential amino acid, is a general phenomenon not limited specifically to methionine(1). The fact that the growth was not fully restored by the added tryptophan is of incidental importance only, and may indicate that yet another essential amino acid was involved, secondarily to tryptophan.

In the experiment conducted with the diet of Hill, *et al.* it is evident that the addition of the amino acid supplements did increase growth rate (Table II). In this case the amino acid supplements were almost as effective as the destruction of the growth inhibitor by heat. It would appear that no essential amino acids other than methionine and arginine were at marginal or slightly deficient levels. Unfortunately, growth data were given by Hill *et al.* for only the first 7 days of the chick's life. Growth data were not provided for a second series of tests conducted with a lower protein level, but it was stated that the results supported those in the first series. It is well known that for at least half of this time, the baby chick is still assimilating the unabsorbed yolk. Experimental diets during this period could not be expected to show very consistent differences since a large part of the total nutrients (unabsorbed yolk) is not controllable. Aside from the shortness of the growth period employed by Hill *et al.*, it is possible that their failure to observe a growth stimulation on the addition of amino acids to a diet containing raw soybeans may have been a manifestation of a very general principle, which holds in amino acid nutrition, as well as elsewhere. Hill *et al.* added 11 amino acids in balanced proportions intended to meet all requirements. The principle involved in this case is that adding a balance to an imbalance does not correct the latter. An imbalance may be corrected only by adding the specific components that are inadequately provided for balance, or by adding a complementary imbalance.

The hypothesis that impairment of proteolysis in the gut is a primary factor which appears to accentuate partial amino acid deficiencies or to convert a marginal supply of an amino acid to a deficiency in effect, has been discussed elsewhere(1,5,6). Recently, it has been shown that highly purified trypsin in the diet will reverse the effects of the raw soybean growth inhibitor, in support of the view that an anti-tryptic action is the primary mechanism of the increase in growth inhibition in this case(7).

Summary. 1. The presence of raw soybean protein in the chick diet was found to develop or accentuate a deficiency of tryptophan in the case of a chick diet marginal in this amino acid. 2. A diet deficient or marginal in arginine and methionine was rendered more acutely deficient when the soybean protein component was present as raw meal. 3. The effects of these accentuated deficiencies on growth were reversed by adding the specific amino acids involved or by heat destruction of the growth inhibitor. 4. These results are in agreement with previous results in the cases of methionine, lysine, arginine and isoleucine.

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Received September 21, 1953. P.S.E.B.M., 1953, v84.

Level of Liver Glycogen in Rats Having Steroid Diabetes. (20638)

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It has been generally believed that a deficiency of insulin limits the storage of glycogen in liver(1). This is not true of the rat having moderate alloxan diabetes(2) although it is noted in severely diabetic animals. The relationship of insulin action to the storage of liver glycogen requires further study with careful exploration of each experimental variable. Many studies employ the level of blood glucose as the sole criterion of the degree of diabetes. This is inadequate for the purpose. The amount of urinary glucose as related to

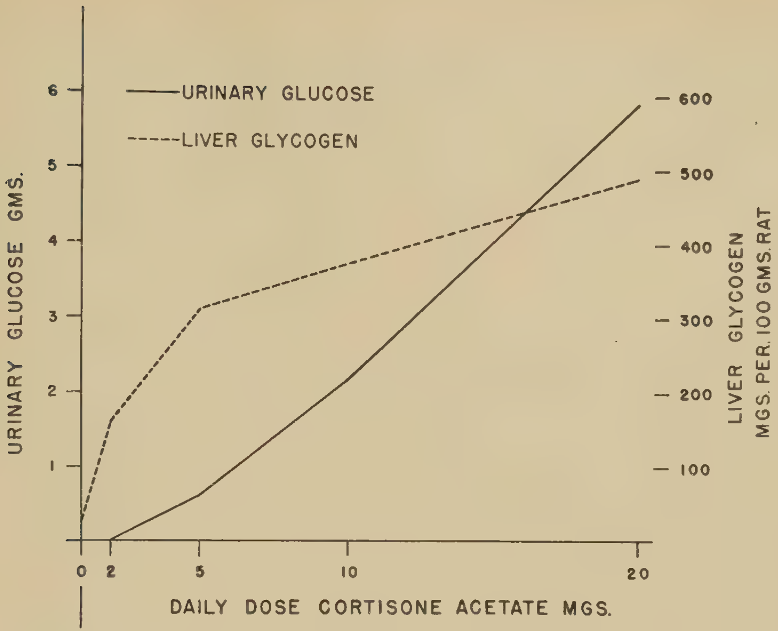

FIG. 1. Levels of urinary glucose and of liver glycogen as related to the dose of cortisone acetate. Averages.

food-intake permits a more quantitative appraisal of the severity of the diabetes.

The positive effect of cortisone and related steroids upon the level of liver glycogen is established. What is the level of liver glycogen in animals having steroid diabetes? Does the animal excrete glucose only after it has reached the limit of its capacity for storage, or does the administration of diabetogenic amounts of steroids interfere with glycogen storage? We find a very high level of liver glycogen in rats having steroid diabetes.

Methods. Male rats of the Sprague-Dawley strain having initial weights of approximately 300 g were placed in metabolism cages and fed 13 ml of a medium carbohydrate diet(3) by stomach tube each morning and late afternoon. The temperature was 74° to 78°C. Twenty-four-hour samples of urine were collected at 8:00 to 8:30 a.m. and were preserved with toluene for the quantitative determination of glucose(4). A micro-suspension of cortisone acetate was injected subcutaneously following each morning feeding. At the end of the injection period each rat was anesthetized with cyclopal; the liver was rapidly excised, weighed and dropped into hot KOH. Glycogen was determined by the use of anthrone(5).

Experiments and results. In Exp. 1 the rats were injected with cortisone for 7 days and the liver excised at the end of a 24-hour fast. The dosage was 2 mg per rat per day, 6 rats; 5 mg, 13 rats; 10 mg, 18 rats; and 20 mg, 16 rats of which 3 died. The dose of 2 mg per rat per day did not cause glycosuria but all of the rats on higher doses excreted measurable amounts of glucose. With each increase in dosage of cortisone there was a significant increase in the average level of liver glycogen and of urinary glucose (Fig. 1). The values for liver glycogen are expressed in relationship to body-weight. Cortisone causes a loss of weight to an extent which is proportional to the dose. These differences in weight loss by 7 days are small and do not significantly effect the shape of the dose-response curve for liver glycogen.

In Exp. 2 the level of liver glycogen was determined in rats which had received 7 daily injections of cortisone. In some of the rats the livers were removed either 4 or 6 hours after the last feeding. The effect of feeding upon the level of liver glycogen is maximal at about

TABLE I. Liver Glycogen of Fasted and Non-Fasted Rats Given Cortisone Acetate.

Daily dose cortisone, mg	Hr post-feeding	No. rats	Days on cortisone	Avg liver glycogen, % of liver wt
5	24	6	7	7.17
	4	2	6⅙	9.23
10	24	6	7	8.06
	6	4	6¼	9.97
20	24	8	7	9.06
	6	7	6¼	8.92

this time. In the remaining animals the livers were removed 24 hours after feeding. Although the numbers of animals are small, the data (Table I) do suffice to show that the peak level of liver glycogen in rats treated with large doses of cortisone acetate is about as high in the fasted as in the fed rat. The highest individual value was 11.84% glycogen in the liver of a fed rat given 10 mg of cortisone acetate daily for 7 days.

Discussion. Liver glycogen is very high in rats having steroid diabetes induced by cortisone acetate. At each higher dose there is an increase in both the amounts of urinary glucose and of liver glycogen. However, the slopes of the dose-response curves for urinary glucose and for liver glycogen are not parallel and at any given dosage level the correlations between the 2 values for individual animals are low. We have not measured these 2 indices of altered carbohydrate metabolism under identical conditions. Urinary glucose is excreted following each feeding and the urine is sugar-free by the end of a 24-hour fast. The deposition of liver glycogen is cyclic. If it were important to study more closely the interrelationship between these 2 indices it would be necessary to measure them simultaneously and at frequent intervals following feeding and during fasting.

Summary. Normal male rats were force-fed a medium carbohydrate diet. Steroid diabetes was induced by the administration of cortisone acetate in doses of 2, 5, 10, and 20 mg/rat/day for 7 days. The level of liver glycogen reached very high values in both fed and fasted animals. The glycogen level was proportional to the dose of cortisone acetate and was correlated with the level of urinary glucose.

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Received September 21, 1953. P.S.E.B.M., 1953, v84.

Excretion of Urinary Mucoprotein Following Parathyroid Extract in Rats.* (20639)

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Following the administration of parathyroid extract to rats, the levels of serum mucoprotein rise(1). When doses of 1000 or more units of the hormone are given over a short period of time, striking changes are seen in the kidneys. The tubules become filled with a water-soluble, alcohol-insoluble material which gives a positive periodic acid-leucofuchsin reaction, and mucoprotein granules appear in tubular cells. These observations suggested that in such animals the urinary mucoprotein levels also might be enhanced.

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* This work was supported in part by grants from the Department of the Army, Surgeon General's Office, from the American Cancer Society, recommended by the Committee on Growth of the National Research Council, and from the Graduate School, University of Illinois.

TABLE I. Urinary Mucoprotein in Rats: Amount of urinary mucoprotein excreted before and after injection of 1000 units of parathyroid extract (Parathormone, Lilly), expressed as mg of mannose-galactose standard.

	Animal No.												
	1	2	3	4	5	6	7	8	9	10	11	12	Mean
72 hr pre-inj.	1.37	.29	.78	.35	1.44*	1.11	.85	1.43	.63	.76	.82	1.92	1.08
72 hr post-inj.	2.52	2.00	2.25	.52	3.87*	2.63	2.47	3.66	2.83	3.52	4.52	1.35	2.84

* Urine collections were made for 48-hr period only.

A mucoprotein has been demonstrated in normal human urine by Tamm and Horsfall (2,3). Their method of precipitating this substance at a concentration of 0.58 M NaCl was applied to rat urines collected before and after the administration of parathyroid extract.

Methods. Twelve young adult female rats (Sprague-Dawley) were housed in individual metabolism cages for the collection of urine without fecal contamination. Urines were collected for a 3-day control period. The animals were fed outside their cages during a one-hour daily period to avoid contamination of the urine samples with food, and water was given *ad lib.* A few days after the control period, animals were injected with 1000 units of parathyroid extract† in 2 divided doses over 6 hours. Urine collections were begun after the last injection and continued for a 3-day period interrupted by daily feeding periods of one hour. Urine collections were taken daily for mucoprotein separation. *Separation and determination of urinary mucoproteins.* Each daily sample of urine was filtered and brought to a final volume of 50 ml by washing the filter paper and residue with distilled water. To the filtrate was added 1.8 g NaCl, to give a concentration of 0.58 M, and the mixture was placed in the refrigerator overnight, when mucoprotein flocculated; the mixture was centrifugalized and the supernatant discarded. The sediment was dissolved in 6 ml of 5% Na_2CO_3 to which a few drops of normal NaOH had been added. After standing for one hour, an aliquot was taken for estimation of the carbohydrate content using the orcinol reagent and a standard containing equal amounts of mannose and galactose.

Results. (Table I). During a 72-hour control period, rats excreted a total of 0.35-1.92 mg (mean: 1.08 mg) mucoprotein carbohydrate determined as mannose-galactose. Using each animal as its own control, an elevation was found in 11 out of 12 animals following parathyroid extract. For a 72-hour period following injection, the excretion was 0.52-4.52 mg (mean: 2.84 mg). The difference was highly significant.

Discussion. Current opinion is divided as to the origin and significance of the plasma mucoproteins. The problem has been recently discussed in some detail(4). The origin of the urinary mucoproteins has, so far as we know, been scarcely considered, although the consensus is that the urinary proteins are excreted through the glomeruli(5). We have inclined to the view that increases in plasma mucoproteins in certain specific situations might be related to the formation of water soluble fractions from the ground substance of connective tissues(6-9,1). Comparable connective tissue changes may underlie even some of the so-called non-specific situations where blood mucoproteins become increased in amount, and a similar explanation would then apply to them. Following parathyroid hormone injection, the following events are noted: dissolution of the bone matrix and the formation of water soluble glycoprotein moieties as demonstrated by direct chemical analysis of frozen-dried tissues; increase in plasma mucoproteins; kidney changes suggesting excretion (and possible reabsorption) of mucoproteins; finally, as shown here, a doubling or trebling of the urinary mucoprotein output. While a part of the normal complement of urinary mucoprotein may be derived from the urinary tract, the above sequence appears to associate at least the additional mucoprotein excreted with events occurring at some point proximal to the urinary tract. To define a causal relationship will entail a greatly expanded knowledge of the tissue mucoproteins and their breakdown products. Meanwhile this study suggests that quantitative urinary mucoprotein studies might profitably be undertaken in a variety of diseases involving connective tissues, including especially the acute and chronic bone dystrophies.

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† Parathormone generously supplied by Eli Lilly Co., Indianapolis, Ind.

Summary. Control rats excrete, in a 72-hour period, 0.35-1.92 mg (mean: 1.08 mg) urinary mucoprotein determined as mannose-galactose standard. Following injection of 1000 units of parathyroid hormone, mucoprotein excretion increased significantly to 0.52-4.52 mg (mean: 2.84 mg). This increase is correlated in time with a dissolution of bone matrix, with an increase in plasma mucoprotein, and with the appearance of mucoprotein granules in kidney tubule cells and mucoprotein-containing casts in tubules.

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Received September 21, 1953. P.S.E.B.M., 1953, v84.

Use of an Organic Chelating Agent in Histochemical Study of Alkaline Phosphatase Activation.* (20640)

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Ethylene diamine tetraacetic acid (EDTA), by virtue of its capacity to form stable chelate complexes with many bivalent metals, will inhibit a number of enzymes *in vitro* presumably through competition with the enzyme for the activating metallic component(1-4). Histochemical application of this property was suggested by the solubility of the chelates formed, and alkaline phosphatase was chosen as a test enzyme for several reasons. 1) It has an ionically available activating metal (magnesium). 2) The enzyme has been extensively studied *in vitro* and considerable data has been accumulated with respect to its activation and inhibition. It provided, therefore, a reasonably satisfactory baseline for the method. 3) At least one component of the system is relatively resistant to alcohol fixation and paraffin embedding. 4) It can be readily demonstrated by 2 different histochemical technics which give identical localizations. 5) Alkaline phosphatase (phosphomonoesterase I) is part of an isodynamic enzyme system and there is reason to suspect that it may actually represent a group of enzymes with a similar pH optimum rather than an entity. There was a possibility, therefore, that these might be separable histochemically on the basis of their potential metallic activators and anionic inhibitors as had been demonstrated *in vitro* in the case of members of the system having different pH optima(5,6).

Method. Rabbit kidney which contains large amounts of alkaline phosphatase in characteristic distribution was used as a test tissue. Thin pieces of fresh kidney were fixed in cold 80% alcohol for 24 hours, dehydrated in cold

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* This work was supported in part by a grant from the U. S. Public Health Service and by the John R. Stark Memorial Fund.

graded alcohol and n-butanol in a period of 6 hours, and then left in cold n-butanol overnight. The tissue was then infiltrated in several changes of paraffin within a period of 2 to 3 hours at a temperature not exceeding 56°C. After embedding, the blocks were refrigerated until ready to cut. Sections were cut evenly at 7 μ and heating was omitted. Such sections, when stored in the refrigerator, retained their activity for many months. Following rapid removal of paraffin in xylol and rehydration in graded alcohols to water, the sections were washed in running water and exposed for 24 hours at room temperature to a solution containing 10 mg per ml of chelating agent adjusted with sodium hydroxide to pH 6.0 using the glass electrode. The concentration of active chelating agent was determined following adjustment of pH by means of a modified Schwarzenbach procedure using standard 0.01 molar calcium acetate with borate buffer at pH 9.2 and eriochrome black T as an indicator (7-9). Appropriate dilution was then made and the final pH rechecked. Following exposure to EDTA, the sections were washed in running water for 5 minutes in order to free the tissue of soluble chelates and excess chelating agent. They were then rinsed in distilled water and placed for one hour, together with active control sections, in a glycerophosphate substrate containing 0.3% sodium glycerophosphate (Eastman) and 1% calcium chloride in 0.05 molar sodium barbital buffer at pH 9.2. Magnesium salts were omitted from the substrate. The sites of alkaline phosphatase activity were then made visible by means of the Gomori cobalt sulfide technic(10), a method which lends itself rather readily to rough semiquantitative standardization. Although the calcium ion present in the substrate is capable of reactivating the enzyme, Ca^{++} is not a strong activator and no discernible evidence of reactivation was noted in less than 2 hours of incubation on numerous occasions. The alternative diazo method(10, 11) was frequently used for confirmation. Reactivation of the system could be readily accomplished by exposure of the slides before incubation in substrate to solutions of various activating ions for 2 to 6 hours or longer. The optimal concentration of activating ion varies widely with the metal used, but, in the case of rabbit kidney, little difference could be detected in concentrations from 0.1 to 0.01 molar, and the former concentration was used routinely in these preliminary experiments. Since the laboratory distilled water contained significant quantities of contaminating ions capable of reactivating or inhibiting the enzyme, all the distilled water used in these experiments was passed through an anion-cation exchange resin. In addition, relatively high concentrations of the test ions were used in order to reduce to a minimum the possible effects of any contaminating cations that might remain in the water or be present in the samples of metallic salts available. The metallic salts used were chlorides or, in a few instances, nitrates of the highest grade of purity available. With few exceptions (*e.g.* beryllium nitrate about pH 4.0) the pH of the 0.1 molar solutions ranged from 5.9 to 6.9. Inactivated and untreated active control sections were run with each experiment.

Observations. Preliminary studies were aimed at determination of optimal levels of pH, temperature, concentration and time of exposure necessary for EDTA inactivation of rabbit kidney alkaline phosphatase.

pH. Ethylenediamine tetraacetic acid, although itself highly insoluble, is capable of reacting with up to 4 equivalents of base to form increasingly soluble salts over a pH range from 2.2 to 10.6. By the use of increasing amounts of sodium hydroxide, therefore, it was possible to adjust the pH of the chelating agent to any desired level. Buffering was not ordinarily necessary since the amount of ion available for chelation in the sections as compared with the large excess of chelating agent present was insufficient to produce significant pH alteration. The efficiency with which any given cation is complexed varies with the pH, and calcium and magnesium are most effectively bound at alkaline pH levels. In view of the fact, however, that effective complexing of these and many other cations will occur on the acid side as well, and that alkaline phosphatase is much more stable on the acid than on the alkaline side, a pH of 6.0-6.5 was found to be optimal in the presence of a considerable excess of the chelating agent. Sections exposed to

TABLE I. Effect of EDTA Concentration on Speed of Inactivation of Alkaline Phosphatase.

	24 hr		90 hr	
EDTA, mg/cc	pH 5.9	pH 8.	pH 5.9	pH 8.
.1	2+	4+	0	2+
.2	+	3+	0	+
.4	±	2+	0	±
.6	0	+	0	±
1.	0	0	0	0
2.	0	0	0	0
5.	0	0	0	0
10.	0	0	0	0
Mg^{++} reactivated	3+	3+	3+	3+
Untreated control	4+	4+	4+	4+

1% EDTA for as long as one month and then reactivated by Mg^{++} showed no appreciable loss of activity on the acid side of neutrality above pH 4.6. Irreversible loss of activity occurred on the alkaline side, however, increasing as the pH optimum of the enzyme was approached.

Temperature. The rate of inactivation was slowed at refrigerator temperatures. Since warming for prolonged periods was undesirable, room temperature (22-24°C) was arbitrarily adopted as a safe and convenient level for these experiments. Active control sections kept at room temperature in buffer solutions of pH comparable with that of the chelating agent, and for comparable periods of time, showed no significant loss of alkaline phosphates activity.

Concentration of chelating agent and period of exposure. As might be expected, the rate of inactivation at any given pH level and temperature was found to be dependent on the concentration of active chelating agent and the period of exposure. Inactivation could be effected in a 24-hour period by concentrations considerably less than 1% and was actually much more rapid at an acid pH (Table I). All these slides could be readily reactivated, however, and tenfold increases in concentration and exposure time had no demonstrable deleterious effect. As a matter of fact, slides exposed at room temperature to the chelating agent in concentrations of 100 mg per ml (10%) and at the non-optimal pH of 7.2 for a period of 6 weeks showed only slight diminution of activity on reactivation with magnesium

Reactivation of EDTA-inactivated rabbit kidney alkaline phosphatase by metallic ions. The slides in each set of more than 10 experiments were cut serially from the same block, inactivated at the same time, and exposed to 0.1 molar concentrations of the various metallic ions for 6 hours under identical conditions of temperature and concentration. Although there were minor individual variations in the different series, Mg^{++} proved to be the most consistently effective ion capable of reactivating the system. Co^{++} and Zn^{++} were almost equally active, however, with Ca^{++} rather less so. These results correspond with those obtained in *in vitro* studies(5,6). Ni^{++} showed slight but consistent activating potential. Sr^{++} and Ba^{++} which have been considered inhibitory by some investigators (12) produced weak to moderate activation. Active control slides in this group showed no evidence of inhibition. Mn^{++}, Pb^{++} and Cd^{++} in 0.1 molar concentrations showed no tendency to reactivate the system but also failed to inhibit it. Hg^{++}, Be^{++} and Cu^{++} were constant strong inhibitors. The inability of Mn^{++} to reactivate alkaline phosphatase by this method is noteworthy, since manganese is reported to be capable of activating the purified enzyme *in vitro*(5,6).

Preliminary studies on kidney tissue from other animals have shown variations in the levels at which certain inhibitory ions can compete for the enzyme under these conditions. In contrast to rabbit kidney alkaline phosphatase, that of guinea pig or monkey kidney is markedly to completely inhibited by Cd^{++} and Pb^{++} in 0.1 molar and slightly inhibited in 0.01 molar concentrations. As might be expected, neither of these ions will activate the EDTA-inactivated system in these tissues. On the other hand, Mn^{++} in 0.1 molar concentration shows no tendency to inhibit the enzyme of any of these tissues and also consistently fails to activate it.

Microscopic examination of the control sections and of those EDTA-inhibited sections reactivated by exposure to the various metallic ions thus far tested has shown no significant variation in localization of alkaline phosphatase activity. It is possible, however, that important components of the alkaline phosphatase system are lost by the method of fixation and embedding used since there is no question that such treatment considerably reduces the intensity of the reaction(10,11). Studies using more efficient methods of tissue preparation such as freezing-drying are continuing. Other components of the phosphatase system are also being studied, and the reversible inactivation by EDTA of 5-nucleotidase has already been demonstrated by histochemical means.

Summary. A method of controlled inactivation of alkaline phosphatase in tissue sections by removal of the activating metallic component through chelation is described. The enzyme activity of alcohol-fixed, paraffin-embedded rabbit kidney can be completely inhibited by EDTA and subsequently reactivated by a variety of metallic cations in 0.1 molar concentration, including Mg^{++}, Co^{++}, Zn^{++}, Ca^{++}, Sr^{++} and Ba^{++}. Ni^{++} is a weak reactivator. The localization of enzyme activity in the tissue is the same regardless of the reactivating ion. Hg^{++}, Be^{++}, and Cu^{++} are constant strong inhibitors. Mn^{++}, Pb^{++} and Cd^{++} fail to reverse EDTA inhibition but will not themselves inhibit the active enzyme in the concentrations used. Preliminary experiments indicate, however, that the alkaline phosphatase of guinea pig and monkey kidney is inhibited by Pb^{++} and Cd^{++} in much lower concentrations.

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Received September 21, 1953. P.S.E.B.M., 1953, v84.

Effect of Colchicine on Synthesis of Desoxyribonucleic Acid in Tissue Cultured Rat Fibroblasts.* (20641)

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Early observations on the effect of colchicine on growing tissue (Dustin(1)) suggested that this substance exerts a stimulatory effect on the process of cell division. In later analyses it was shown by Ludford(2) and others that the increase in mitotic figures was the result of an inhibition of mitosis at metaphase. Ludford postulated an inhibitory effect on the mitotic spindle, which has recently been convincingly illustrated by the work of Inoue(3) on the birefringence of the spindles of normal and colchicine-treated sea urchin eggs. The suggestion of a specific effect led to experiments which demonstrated that at low col-

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* This investigation was supported by research grants from the National Cancer Institute of the National Institutes of Health, Public Health Service, the Damon Runyon Memorial Fund, and the Jane Coffin Childs Fund and was carried out during tenure of a Fellowship in Cancer Research of the American Cancer Society, recommended by the Committee on Growth. The author is indebted to Dr. Gabriel C. Godman for his valuable suggestions, and to Dr. Margaret R. Murray for the use of her laboratory facilities. The technical assistance of Miss Priscilla Goettler is gratefully acknowledged.

chicine concentrations sufficient to cause mitotic inhibition, other metabolic processes which are not directly concerned with the mechanics of cell division can proceed. For example, Zeuthen(4) reported that although cleavage of echinoderm eggs is inhibited during colchicine treatment, the rhythmic behavior of oxygen uptake and nuclear refractivity which normally occur during cleavage, also occur during colchicine blocking. Skipper *et al.*(5) showed a continuous incorporation of C^{14} into nucleic acids of tumor cells during colchicine treatment. The production of polypoid cells with colchicine (Blakeslee and Avery(6)) gives evidence that this substance does not directly interfere with the synthesis of chromosomal material. This author has observed a few meristematic cells from the buds of Crepis, whose normal diploid complement is 6, with more than 100 chromosomes after a few days of colchicine treatment. The presence of cells with higher than the tetraploid number can only be due to continued synthesis during treatment.

Preliminary experiments have shown that the individual nuclei of growing cultures which have been treated with colchicine for approximately one day, invariably contain higher amounts of desoxyribonucleic acid (DNA) than untreated controls. The following experiments were designed to trace the course of DNA synthesis during colchicine treatment.

Materials and methods. Fibroblast cultures obtained from explants of the subcutaneous fascia of a 5-day-old rat were grown on Maximow slides for about 7 days. This tissue affords a source of fibroblasts in which the division rate is relatively steady, and which are relatively genetically pure with regard to tissue type (Murray *et al.*(7)). The cultures were treated with colchicine in the feeding solution at a concentration of 10^{-5} molar. After treatment they were fixed at various intervals along with controls in neutralized 50% formalin, followed by washing for 3 hours in running tap water, and 15 minutes in 80% ethanol to remove excess formaldehyde. They were then stained according to the Feulgen procedure using a hydrolysis time of 15 minutes. Fifty to 60 randomly selected interphase nuclei, distributed among 3 explants, were measured from each culture, totaling approximately 400. One culture was used for each treatment, and 2 for the controls. The intensity of stain was determined by absorption measurements using a microspectrophotometer of the type described by Pollister and Ris(8), as modified by Moses(9). The slides were mounted in oil with a refractive index of 1.548, approximately that of the stained cells, to minimize light scattering. A wave-length of 565 mμ was used, which lies at the absorption peak of the stain. Relative amounts of DNA in the individual nuclei were calculated by multiplying the extinction by the nuclear area. The latter was approximated by direct measurement of the 2 diameters of the flattened elliptical nuclei with an ocular micrometer. The mitotic indices are the fractions of cells found in mitosis and were determined by counting up to 1000 nuclei in randomly selected fields. Fewer nuclei, approximately 500, were counted in the cultures showing very high indices.

Results. The upper curve in Fig. 1 shows a typical distribution curve of the amounts of DNA in the individual nuclei of a young growing culture. The first peak, which predominates, consists of nuclei containing the normal diploid amount. The nuclei in the second peak have doubled their DNA, presumably during interphase (Swift(10)), and are about to undergo division. This curve is in accord with the results obtained by Frazer and Davidson(11) on the distribution of DNA in cultured chick fibroblasts. That the cells in the second peak are not true tetraploids is made evident by the curves obtained from older cultures which are no longer growing. In such cultures the second peaks are almost non-existent. Henceforth Swift's(12) designation: Class 2, 4, 8, . . . pertaining to nuclei with diploid, tetraploid, octoploid, . . . amounts of DNA will be used in describing the various classes. The lower curves show the shifting of the class distributions with time in colchicine. After 10 hours of treatment, the ratio of class 4 to class 2 nuclei increases, and some class 8 nuclei appear. At 21 hours the class 4 predominate and class 8

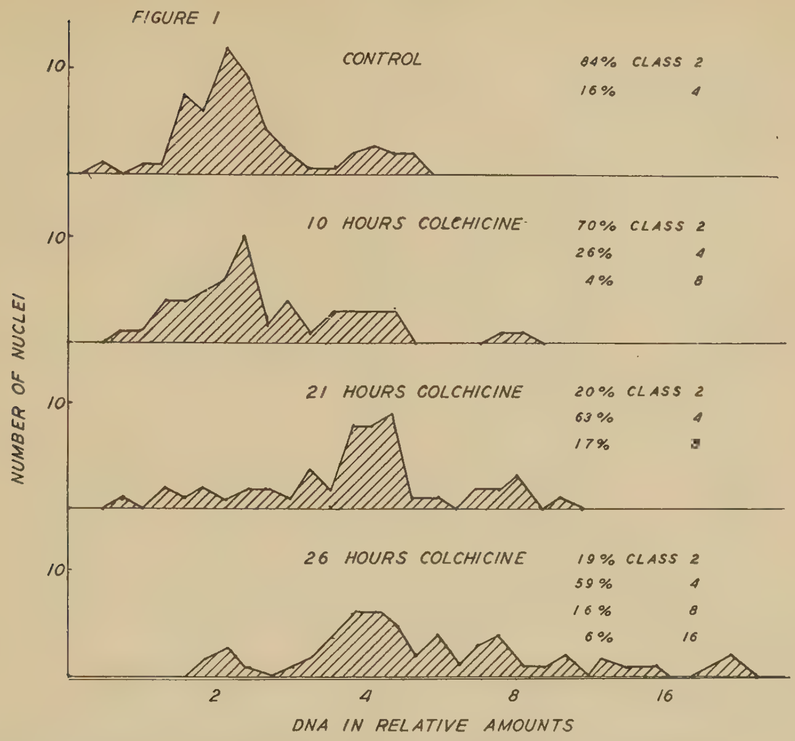

FIG. 1. Effect of time of colchicine treatment on frequency distributions of DNA per nucleus.

are numerous. At 26 hours even some class 16 nuclei are found. The boundaries of the various classes were established by multiplying the values obtained for the boundary between the first 2 peaks by the appropriate integers. The minimum between peaks 2 and 4 became very obvious after constructing the distribution curve for the pooled nuclei of all the cultures. After 21 hours, a progressively increasing number of multinucleate cells makes any attempt to determine relative numbers of nuclei in the various classes difficult. Most of these apparently belong to the higher classes, although the errors involved in adding quantitatively determined values of the micronuclei are sufficiently great to preclude their assignment to any definite class. These cells amount to approximately 1% at 17 hours, 5% at 21 hours, and 30% at 26 hours. In determining the class distributions the multinucleate cells were eliminated, resulting in a slight underestimation of the degree of polyploidy as time of treatment increased.

In accord with previous investigations, the mitotic index was found to rise irregularly during the first 24 hours of treatment, after which it declined in the same manner. Continuous observation of several fields during the first 24 hours showed that cells in "colchicine mitosis" revert to the resting state during this period.

Fig. 2 shows the changing mitotic index with time, and also the changing "degree of polyploidy." (The use of the latter term does not presume to distinguish between true polyploids and nuclei of lower classes with increased DNA.) The degree of polyploidy was calculated by assuming the mitotic nuclei to be distributed among classes 4, 8, and 16 in the same ratio as the interphase nuclei, and by multiplying the adjusted (to include mitotic nuclei) percentages in the various classes by their class value. Thus degree of polyploidy, D, equals:

$$\frac{I}{N_T - N_2}(4N_4 + 8N_8 + \ldots) + \frac{1 - I}{N_T}(2N_2 + 4N_4 + \ldots)$$

where I is the mitotic index, N_X the number of nuclei in class x, and N_T the total number

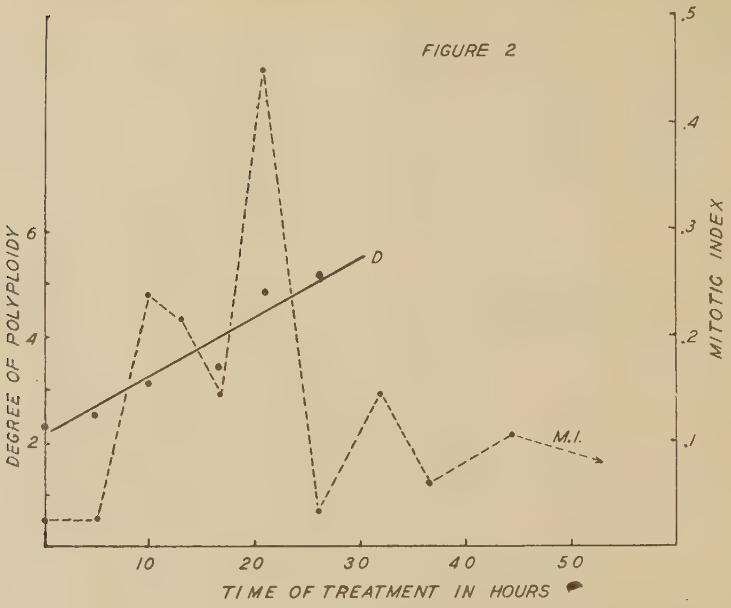

FIG. 2. Effect of time of colchicine treatment on mitotic index and "degree of polyploidy."

of nuclei. D would be 2 in a normal non-growing culture, between 2 and 4 in a growing culture, and 8 in a hypothetical homogeneous octoploid population. The assumption that the proportions among the higher classes is the same in the mitotic nuclei as in the resting is an approximation which is sufficient for the purpose to which it is to be put. It can be seen from Fig. 2 that the degree of polyploidy approximately doubles during the first 18 hours. The coefficient of correlation between D and time during this period, was calculated to be .966. The probability that such a coefficient would be obtained where no correlation exists, is less than 1%. Assuming that during this period the number of cell divisions of any kind is small, the total number of cells therefore remaining the same, the increase in D will be proportional to the increase in the DNA of the culture. Hence DNA approximately doubles during this period. This is at variance with the results of Davison *et al.*(13) who have reported a slight decrease of total DNA phosphorus after colchicine treatment, using chemical extraction technics on colchicined cultures.

According to Lambert and Hanes(14), the average period between the onset of prophase and the end of telophase in cultured rat fibroblasts is about 35 minutes at 37°C. A similar figure was obtained for chick fibroblasts (Hoffman(15)). The amount of time spent in any one phase of mitosis would be proportional to the number of cells found in that particular stage. Therefore the mitotic index of .027 for the control cultures indicates that the average cell completes the division cycle in about 22 hours. The typical interphase period has been found to be approximately 9 hours (Fell and Hughes(16)) by direct measurement of the length of time between successive mitoses. However, the 22 hours arrived at here includes in the average, cells which are not actively dividing. During this 22-hour period the number of cells, hence total DNA for the control cultures, also approximately doubles. Considering the approximations used, the agreement between these 2 figures, 18 hours for the colchicined cultures and 22 hours for the controls, is as good as could be expected. It can be stated with assurance that the increase in the total DNA over the first 26-hour periods is approximately the same in the colchicine treated cultures as in the controls, and colchicine is not apparently directly concerned with DNA synthesis as such.

After the first day, there appears to be an

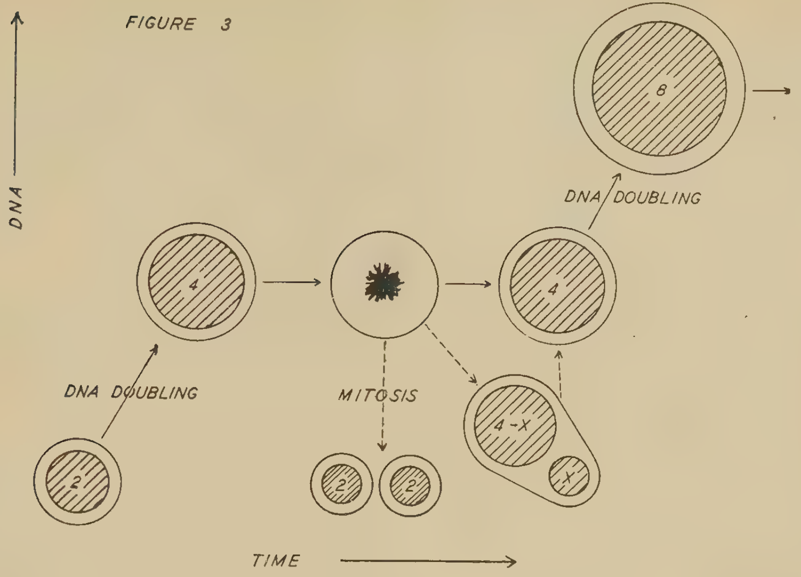

FIG. 3. Possible scheme for production of higher DNA class cells during colchicine treatment.

eventual diminution of activity, as indicated by the decreased mitotic index. Subsequent growth of the cultures is slight as compared by inspection with the controls. Whether this decreased growth is due to a general toxicity of the colchicine, or to production of genetically unbalanced cells is not certain. Interestingly enough, colchicine had no apparent effect on the single peaked distribution curves of non-growing cultures. This is in accord with the long established fact that colchicine in these low concentrations does not drastically disturb the resting cells of adult differentiated tissues.

Fig. 3 illustrates a possible scheme to explain the production of the various class nuclei under the influence of colchicine. The majority of cells probably follow the unbroken line leading through DNA duplication to the metaphase like figure typical of colchicine treated cells. From this point, several pathways might be open. One is through resumption of mitosis, a "colchicine escape," especially after prolonged treatment. Earlier, the most likely pathway is directly back to the interphase, maintaining the class 4 amount of DNA. A more devious route would be through separation of the chromosomes to form a multinucleate cell, thence restitution to the mononucleate class 4 stage. This whole process could be repeated forming the class 8 and 16 nuclei.

Summary. 1. In spite of its action in blocking mitosis, colchicine does not directly inhibit the synthesis of DNA. During the first 26 hours of treatment, DNA synthesis proceeds at the same rate as in untreated controls. 2. The result of colchicine treatment is a progressive increase in the number of cells belonging to the higher DNA classes, up to 26 hours. 3. Prolonged treatment eventually results in decreased growth.

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Received September 24, 1953. P.S.E.B.M., 1953, v84.

Filter Paper Electrophoresis of Serum Proteins of the Domestic Fowl.*

(20642)

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(Introduced by C. P. Leblond.)

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Filter paper electrophoresis(1,2) of avian serum proteins has demonstrated (a) the separation of albumin, 2 α-globulins, a β-globulin, and a γ-globulin from the sera of cockerels and immature pullets; and (b) the appearance of a presumptive lipovitellin (PLV) fraction in the sera of laying hens or estrogenized immature pullets(3). It has further been shown(3) that electrophoresis in a veronal buffer made up with 20% methanol results in the separation of a new slow-moving fraction from the sera of estrogenized immature pullets or laying hens. This fraction is not present in comparable electropherograms of sera of immature pullets; it is poorer in lipid than the PLV fraction and rich in phosphorus. For convenience this fraction has been designated presumptive phosphoprotein (PP).

The following additional observations are reported in the present communication: (a) the presence of a third α-globulin fraction on electropherograms of fowl sera; (b) the application of the Feulgen plasmalogen reaction to the electropherograms; (c) the double nature of the PP fraction; and (d) the results of studies using P^{32} labeled inorganic phosphate.

Methods. Electrophoresis was performed as described previously(3) with the exception that the apparatus was kept in a cold room at 6° to 7°C. At this lower temperature it was necessary to raise the potential to 200 volts in order to obtain 8 to 9 ma. with filter paper 17 inches in width and 16 inches in length and run in methanolic buffer. This lower temperature has given closer reproducibility. It is specially recommended if methanolic buffer is being used.

Experimental results. The third α-globulin was first observed when making quantitative determinations of the protein fractions by eluting them from the electropherograms with 1% NaCl and applying Weichselbaum's biuret method(4) to the eluates. The positions of the fractions were established in the first place by rapidly staining a strip of the paper with Brom Phenol Blue. It was subsequently found that this α_3-globulin may be seen well on papers stained for at least 30 minutes in a saturated solution of Naphthalene Black in 60% methanol and then washed in methanol containing 10% glacial acetic acid. It is less readily seen on papers stained with Brom Phenol Blue because it seems that this dyestuff, when associated with α_3-globulin, is relatively easily washed off the papers. However, papers stained with Brom Phenol Blue may subsequently be restained with Naphthalene Black. We have in this way successfully restained older electropherograms on which the original Brom Phenol Blue had faded quite badly. The α_3-globu-

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* Macdonald College Journal Series No. 335. Supported by a Research Grant from the National Research Council of Canada.

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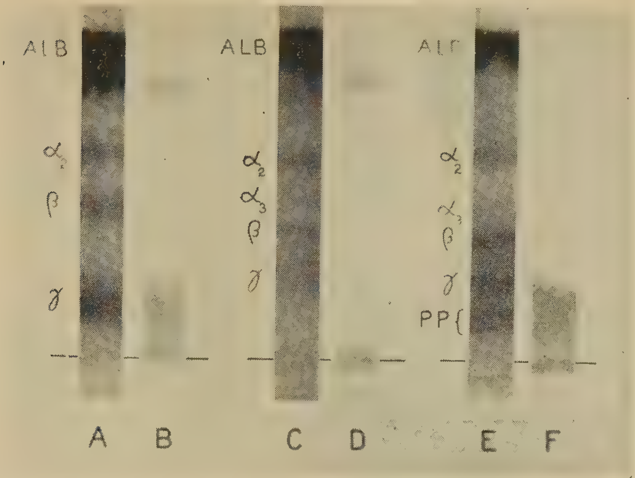

FIG. 1. Electropherograms of human serum (A and B), immature pullet serum (C and D) and serum from estrogenized immature pullet (E and F). Buffer: veronal in 20% methanol, pH 8.6. Papers A, C and E stained with Naphthalene Black; papers B, D and F stained with Oil Red O. Horizontal line marks point of application. Note lipid slightly behind albumin in immature pullet (cp. C and D) and absence of this lipid in the estrogenized pullet (cp. E and F). Note α_3-globulin fraction in C and E, but not in A. Two PP bands are clearly defined in the serum of the estrogenized pullet (E), while lipid has migrated as far as the leading edge of the γ-globulin. Sample of human serum was taken from a subject who had not fasted, hence high lipid content.

lin has been found on electropherograms whether run in aqueous or methanolic buffer. The α_3-globulin is well shown in Fig. 1C and 1E. Both these electropherograms displayed faint narrow α_1-globulin bands situated approximately one-third of the way from the trailing edge of the albumin zone to the leading edge of the α_2 zone, but these α_1 bands were too faint for satisfactory reproduction in Fig. 1.

Staining with Oil Red O(5) and osmic acid (3) has demonstrated the presence of 2 lipid fractions on electropherograms of human sera and sera of immature pullets. The first fraction moved in association with the albumin or somewhat more slowly(5,3). This slower movement is pronounced in 20% $^{v}/_{v}$ methanolic buffer as compared with aqueous buffer (Fig. 1B). Maurer(6) has shown that lipid phosphorus moves in closer association with the albumin the greater the proportion of serum that is included in the buffer. In our experience, electropherograms of sera from estrogenized immature pullets or laying hens have not displayed any lipid staining in the albumin region or behind this region despite the accompanying lipemia (Fig. 1, comparison of D and F). This has been the case whether the buffer were aqueous or methanolic. The second lipid fraction remained at the origin when sera from immature pullets were run in aqueous or methanolic veronal buffer (Fig. 1D). Post-absorptive human sera displayed lipid staining similar to that of unestrogenized immature pullets. However, in the case of a human serum from a non-fasting patient, the second lipid fraction tended to spread between the origin and a position just ahead of the γ-globulin whether the buffer used were aqueous or methanolic (Fig. 1B). These observations agree with those of Kunkel and Slater(7) on electropherograms of human sera run in aqueous veronal buffer. Electropherograms of sera of estrogenized immature pullets or laying hens displayed a great increase of the second lipid component. It has been shown that lipid staining of electropherograms of such sera run in aqueous buffer is associated with a "presumptive lipovitellin" (PLV) component(3). When such sera were run in methanolic buffer, the electropherograms stained with Oil Red O over a zone of practically the same extent as when run in aqueous buffer (Fig. 1F). Specially

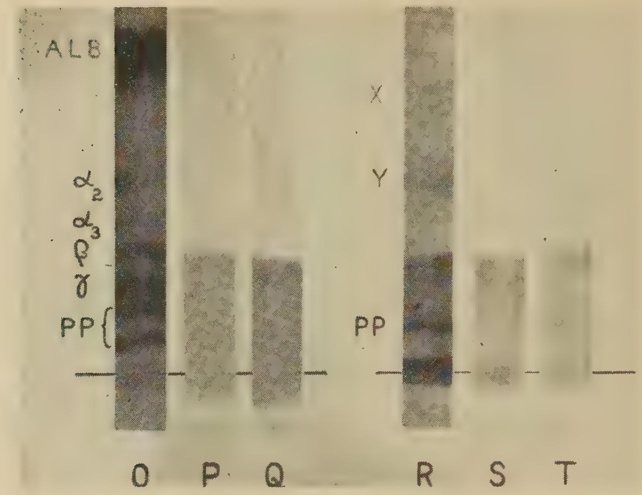

FIG. 2. Electropherograms of sera from estrogenized immature pullet (O, P and Q) and of egg yolk diluted with 1.0% NaCl (R, S and T). Buffer: veronal in 20% methanol, pH 8.6. Papers O and R stained with Naphthalene Black and P and S with Oil Red O. The Feulgen plasmalogen reaction was applied to papers Q and T. Lipid in the serum sample has moved as far as the trailing edge of the β globulin; the two PP fractions are well resolved, and the Feulgen reaction in general corresponds closely with the zone stained with Oil Red O. The yolk electropherograms have stained heavily at the point of application both with Naphthalene Black and with Oil Red O, and display a stronger plasmalogen reaction at the same position. The yolk pattern shows only one definite PP zone. Note the presence of two minor yolk protein fractions (X and Y).

intense staining in the PP region was not apparent when the birds had received 1 mg ODB (estradiol benzoate) intramuscularly on each of 3 successive days. However, when twice this amount of ODB had been given, the PP zones were associated with relatively more intense staining by Oil Red O.

The Feulgen plasmalogen reaction was applied as follows: The paper was dried at 25°C, immersed in 0.05 M mercuric chloride for one minute, washed in water, and then immersed in fuchsin-sulphurous acid reagent. (0.35 g fuchsin and 1.9 g sodium metabisulphite added to 100 ml of 0.15 N HCl and left to stand overnight. Next morning the mixture is shaken to dissolve the fuchsin, decolorized with "Norite" and filtered for use). The reaction (Fig. 1 O,P,Q) gave results corresponding qualitatively to the results obtained by staining with Oil Red O. However, the Feulgen reaction was slightly more intense in zones corresponding in position to the PP zones even when the pullets had received only 2 mg ODB on each of 3 successive days. Duplicate papers extracted with ethanol-ethyl ether (3:1) containing 5% trichloracetic acid, followed by extraction with chloroform-ethanol (1:1) no longer gave the plasmalogen reaction. These observations proved that the acetal phospholipid content of the serum of the fowl is increased in laying hens or estrogenized pullets as compared with control immature pullets. The presence of plasmalogen in egg yolk was first noted by Stepp, Feulgen, and Voit(8). Feulgen and Bersin(9) later estimated that egg phosphatides only contained approximately 0.1% plasmalogen as against 10 to 12% in muscle phosphatides. The amount of plasmalogen in the phospholipid of avian sera is presumably low; Raney *et al.*(10) did not mention its presence in fowl sera. Electropherograms of human sera also gave a faint Feulgen plasmalogen reaction in the regions that stained with Oil Red O. The plasmalogen reaction has a methodological advantage over staining with Oil Red O in so far as it is a reaction rather than a staining procedure. The reported presence of desoxyribonucleic acid in avian sera(11) prompted attempts to demonstrate a positive Feulgen nucleal stain on papers freed from lipid by extraction with alcohol-ether free from trichloracetic acid. These attempts have not been successful, but this may be related to the minute concentrations of DNAP recorded for serum.

A feature of the PP fraction separable on electropherograms prepared with sera of estrogenized pullets or laying hens with methanolic buffer was that this fraction appeared as 2 distinct bands, although these were close together (Fig. 1E). The double nature of the PP fraction tends to be obscured in more heavily estrogenized birds because of "tailing" associated with high lipid content of the sera. The double nature of the PP band was, therefore, partially obscured in electropherograms of sera of estrogenized pullets previously figured by us(3).

The serum of estrogenized pullets was diluted with an equal volume of water, and the resultant precipitate of serum "lipovitellin" was then taken up in 1.0% saline and subjected to electrophoresis in methanolic buffer. The electropherogram displayed a doublet of bands corresponding in mobility to the PP bands of a parallel electropherogram of the original serum run on the same paper.

An electropherogram of egg yolk diluted with 1.0% NaCl and run in methanolic buffer is shown in Fig. 2R, S, and T. The yolk for dilution was carefully withdrawn by syringe from washed intact yolks in order to obviate contamination by egg-white proteins. The origin showed heavy staining with Naphthalene Black and a distinct band (marked PP in Fig. 2R) which probably corresponds to one of the PP bands of Fig. 2O. The dark and wider band in advance of this (Fig. 2R) may correspond to the other PP band of Fig. 2O. The electropherogram of the diluted yolk also showed plainly the presence of a component corresponding in position to the α_2-globulin of serum (marked Y) and of another fainter component corresponding in position to α_1-globulin (marked X).

It seemed possible that the heavy deposit of lipoprotein at the point of application (Fig. 2R and S) might be associated with the physical structure of the yolk emulsion. Accordingly, yolk samples (2 ml) were suspended in 1.0% NaCl and centrifuged. The supernatant was submitted to electrophoresis using methanolic buffer (Fig. 3J and K). The precipitate of lipoprotein was dissolved in 10% NaCl. This solution was then diluted with 10.0% NaCl to the same total volume as the volume of the original suspension in 1.0% NaCl. Filter paper electrophoresis (methanolic buffer) gave the results shown in Fig. 3L and M. The supernatant obviously contained a lipoprotein fraction which occupied a wide zone on the paper, and also contained the X and Y fractions. The precipitate showed a double protein band containing some lipid and corresponding to the PP fractions of sera of estrogenized pullets. There was marked retention of lipid at the point of application but no marked retention there of protein, as had been the case when the simple suspension of egg yolk was examined (Fig. 2R and S). The proteins on paper J represented approximately 1.8 times as much protein as was present on paper L. The relation of these fractions to the lecithovitellin and livetin fractions described in the literature calls for further study.

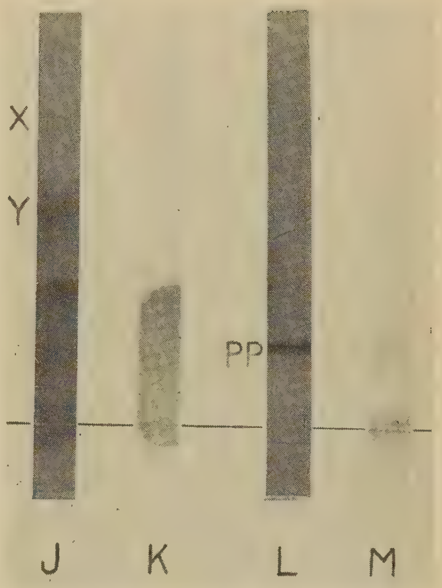

FIG. 3. Electropherograms of supernatant from centrifuging a dispersion of egg yolk in 1% NaCl (J and K) and of a solution prepared by dissolving the precipitate in 10% NaCl and then making up to the original volume with 10% NaCl (L and M). Buffer: veronal in 20% methanol, pH 8.6. Papers J and L stained with Naphthalene Black. Papers K and M stained with Oil Red O.

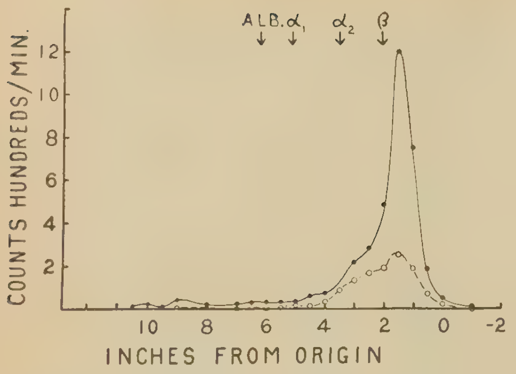

FIG. 4. Distribution of P^{32} on electropherogram of serum of immature pullet (8 wk of age) which had received 8 daily injections of 1 mg ODB. Orthophosphate labeled with P^{32} (63 μc total activity) injected subcutaneously on morning after last ODB injection. Blood drawn 12 hr after injection of radioactive phosphate. Papers scanned through ¼ in. slit under Geiger-Mueller counter. Buffer: aqueous veronal, pH 8.6; ionic strength = 0.05. Full line and dots = total counts; broken line and circles = counts after extraction of lipid.

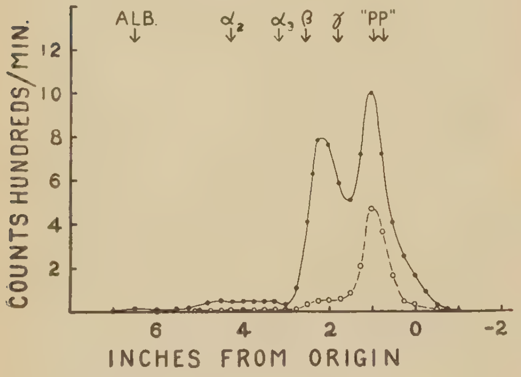

FIG. 5. Distribution of P^{32} on electropherogram of serum of immature pullet (8 wk of age) which had received 3 daily injections of 2 mg ODB. Orthophosphate labeled with P^{32} (68 μc total activity) injected subcutaneously on morning after last ODB injection. Blood drawn 11 hr after injection of radioactive phosphate. Papers scanned through ¼ in. slit under Geiger-Mueller counter. Buffer: 20% methanolic veronal, pH 8.6. Full line and dots = total counts; broken line and circles = counts after extraction of lipid.

Studies using subcutaneous injections of inorganic phosphate labeled with P^{32} gave electropherograms (aqueous buffer) with high counts in the PLV region. Extraction of lipid from these papers as described above and recounting gave considerably lower counts in the PLV region (Fig. 4). When these experiments were repeated using methanolic buffer as previously reported, the new slow-moving band (PP fraction) was associated with a high count (Fig. 5). In a previous report(3) it was mentioned that the counts of the PP band were not appreciably reduced by extraction of lipids, although the counts in the heavy lipid staining region ahead of this were reduced. These earlier experiments, however, were conducted at room temperature. In later work at 6° to 7°C a proportion of the lipid did remain in association with the PP fraction, as may be seen clearly from Fig. 5. The resolution of the counting arrangement was not adequate to determine whether or not the PP zone counted as a double zone.

Discussion. The relation of the results of filter paper electrophoresis of avian sera to the results secured by a number of workers (12-16) by the classical Tiselius apparatus is not at this stage clear. It is possible, however, that further elucidation of the lipids and lipoproteins of avian sera may be facilitated by comparisons of results obtained by both technics. Clegg and Hein(16) have recently described results obtained by subjecting avian sera to the classical electrophoresis in borate buffer of pH 8.6. They distinguished 6 components in sera from untreated chickens and 7 components in sera from estrogenized chickens. The following very tentative correlations between the components described by Clegg and Hein and those here described may be suggested.

Component of Clegg and Hein(16) Control chicken	Component as presently described Immature pullet
2	Albumin
3	α_1 globulin (and lipid ?)
4	α_2 "
?	α_3 "
5	β "
6	γ "

The enormous increase in component 5 as a result of estrogen treatment might then be equated with the fraction which we have designated PLV(3). We have not been able to observe on paper electropherograms any fraction corresponding exactly to component 1 of Clegg and Hein(16), whether the buffer were veronal, borate or diethanolamine. (The latter buffer yields excellent separations but has the disadvantage of interfering with the biuret and Kjeldahl methods of determining protein). In conclusion it should be stressed again that the foregoing correlations of the results by the 2 methods are purely tentative.

Summary. 1. An albumin and 5 globulin fractions have been observed on electropherograms of sera of sexually immature pullets. The albumin fraction of such sera was associated with lipid, but lipid was not observed in association with the albumin of sera of estrogenized pullets. 2. Zones of the electropherograms of sera of pullets that stained with Oil Red O also gave the Feulgen plasmalogen reaction. The Feulgen reaction was stronger with sera of estrogenized birds. 3. Electropherograms of yolk dispersed in saline were prepared using 20% methanolic veronal buffer. A presumptive phosphoprotein fraction was distinguishable on these papers; it resembled closely the presumptive phosphoprotein fraction of sera of laying hens or estrogenized immature pullets. The presumptive phosphoprotein zones were doubled for both yolk and sera. 4. Studies of electropherograms of estrogenized immature pullets that had received injections of phosphate labeled with P^{32} showed that a large moiety of the phospholipid moved ahead of the phosphoprotein fraction when methanolic buffer had been used to separate this PP fraction, although a part of the phospholipid did remain in association with the PP fraction.

The authors thank the National Research Council of Canada for the Research Grant which supported part of this work; Messrs. Schering, Ltd., Montreal, for generous gifts of hormones; and Dr. J. H. Quastel and Dr. C. P. Leblond of McGill University for helpful suggestions.

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Received September 24, 1953. P.S.E.B.M., 1953, v84.

Augmentation of Human Chorionic Gonadotrophin by Plasma and Other Non-Hormonal Substances.* (20643)

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While studying the effects of chorionic gonadotrophin on the human testis, it was observed that the administered hormone could be readily detected in the patients' plasma(1). Furthermore, 24 hours after a single intramuscular injection, the hormonal activity accounted for in the patients' plasma appeared to exceed the total administered dose. Since the method used for assaying chorionic gonadotrophin involved injecting unaltered plasma in rats, it seemed likely that plasma, by some mechanism, enhanced the activity of the hormone as measured in the assay animals. Previous studies have demonstrated that *in vitro* addition of non-hormonal substances, such as eggwhite(2), lemon juice, heme(3), metallic salts(4) and insoluble hydroxides(5), can augment the activity of *pituitary* gonadotrophin preparations, and that these substances probably act by prolonging absorption from the site of injection(4). The experiments described in this report were performed to determine if the phenomenon of augmentation by non-specific materials might also apply to chorionic gonadotrophin and thus account for the surprisingly high levels observed in the plasma of men receiving the hormone.

Methods. Intact female Sprague-Dawley rats, 25 days old at the time of the first injection, were used for assaying chorionic gonadotrophin. All materials to be assayed were administered twice daily for 3 days. Twenty-four hours after the last injection the rats were killed and the uterus and ovaries were weighed. Two preparations of chorionic gonadotrophin were used: a commercial product, "A. P. L.",[†] and the International Standard.[†] The following materials were mixed with chorionic gonadotrophin to determine if they were capable of augmenting hormonal activity: unaltered human plasma, boiled plasma, rat serum, egg-white and polyvinylpyrollidone. The effect of plasma obtained from 3 normal men was compared with that obtained from 2 hypogonadal men, one having primary testicular failure with high urinary gonadotrophin levels, the other having hypopituitarism with undetectable gonadotrophins. Materials were prepared for assay by diluting with water so that the total amount administered to each rat was contained in 6 ml, and 1 ml was administered subcutaneously at each injection, except in experiments involving polyvinylpyrollidone, where the volumes were 1.2 and 0.2 ml, respectively. All preparations were kept at 4°C, except those mixtures that were first incubated at 37°C, as indicated in Table II.

Results. Assay of aqueous solutions of chorionic gonadotrophin: The effects of the commercial product "A. P. L." were entirely similar to results obtained with the International Standard (Table I). Compared with the uninjected controls, 0.5 international units elicited no significant change in uterine weight, 1.0 unit produced an approximate doubling of uterine weight, and 2.0 units gave a maximal response. With these doses, ovarian weight did not increase, but with larger doses of 5.0 to 50.0 units a gradual increase in ovarian weight was observed. The consistency of these dose-response relationships is evidenced by the close agreement of our results with those of others(6-8) who have used the uterine weight response of rats for assaying the International Standard. In view of these findings, uterine weight, rather than ovarian weight, was the criterion employed for measuring the biologic

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* Supported by a grant from Schering Corp. through the courtesy of Dr. E. S. Henderson and by a grant, No. A-121(C), from the Division of Research Grants and Fellowships of the U. S. P. H. Service.

[†] The "A. P. L." used in these studies was supplied by Ayerst, McKenna and Harrison, Ltd. The "International Standard for Chorionic Gonadotrophin" was obtained from the U. S. P. Reference Standards, 4738 Kingsessing Ave., Philadelphia.

TABLE I. Assay of Aqueous Solutions of Chorionic Gonadotrophin.

Chorionic gonadotrophin				
Type	Internat. units	Uterine wt, mg	Ovarian wt, mg	No. of rats
Internat. standard for chorionic gonadotrophin	.5	27	9.3	12
	1.0	52	11.1	10
	2.0	121	12.4	20
Uninj. controls*	—	25	10.5	34
"A. P. L."	.5	36	11.4	12
	1.0	56	10.1	15
	2.0	107	12.1	16
	5.0	137	19.1	7
	10.0	110	21.5	4
	50.0	114	37.1	4
Uninj. controls*	—	30	10.4	27

* Values for uninj. controls also apply to data of Tables II and III.

activity of mixtures of chorionic gonadotrophin and augmenting substances. Accordingly, in the experiments recorded in Table II and III, amounts of materials were selected which produced uterine, but not ovarian, weight increases, and therefore only the values for uterine weight are tabulated.

Human plasma added to chorionic gonadotrophin produced a 5- to 10-fold increase in hormonal activity (Table II). The effects of the hypogonadal patients' plasma were no different than those of plasma samples obtained from normal individuals. When mixed with plasma, as little as 0.2 units of chorionic gonadotrophin produced maximal increases in uterine weight. When an adequate amount of hormone was used, quite small amounts of plasma sufficed to enhance hormonal activity, but when the amount of hormone was reduced below a certain level, increasing the amount of plasma failed to produce an effect. For example, the addition of 0.01 ml of subject L.'s plasma to 0.4 unit of hormone produced clearcut augmentation. With 0.2 unit of hormone, a definite effect was noted from 0.4 ml plasma, but not from 0.04 ml plasma. When the amount of hormone was reduced to 0.1 unit, no effect was observed from increasing the amount of plasma to as much as 3.0 ml.

Incubation of the hormone-plasma mixture for 4 to 20 hours at 37°C resulted in a further increase in potency in one experiment with subject E.'s plasma, but in 2 subsequent trials no effect was noted from incubation using plasma obtained from subjects L. and P. (Table II).

Boiled plasma, rat serum, eggwhite and polyvinylpyrollidone produced definite increases in activity of chorionic gonadotrophin, similar to the effects observed with untreated human plasma (Table III).

Augmentation was not observed when plasma and hormone were kept separate and administered at different subcutaneous sites. No increase in uterine weight occurred from the administration of a plasma-hormone mixture to rats castrated three days prior to the first injection.

Discussion. Although the ability of nonspecific materials to increase the activity of *pituitary* gonadotrophin preparations has been repeatedly demonstrated, most previous studies have failed to demonstrate augmentation of chorionic gonadotrophin. Thus, augmentation was not observed when substances such as plasma(2), heme(3), and zinc and copper salts(4) were added to chorionic gonadotrophin, although under the same experimental conditions these materials produced marked increases in activity of pituitary gonadotrophin preparations. Certain differences in experimental procedure may explain why augmentation of chorionic gonadotrophin, which was regularly observed in our studies, was not seen in earlier investigations. First, in most studies of the augmentation phenomenon, ovarian weight has been used for assaying both pituitary and chorionic gonadotrophins. Although a sensitive and useful

TABLE II. Augmentation of Chorionic Gonadotrophin by Human Plasma.

Patient	Plasma, ml	Chorionic gonadotrophin		Procedure		Uterine wt, mg	No. of rats
		Type	Internat. units	°C	hr		
L., normal ♂	.1	Internat. standard for chorionic gonadotrophin	.1	4		38	4
	3.0		.1	4		25	4
	.04		.2	4		30	4
	.4		.2	4		88	4
	.01		.4	4		67	4
	.04		.4	4		89	4
	.2		.1	37	4	25	4
	3.0		0	4		28	4
P., normal ♂	.1	A. P. L.	.1	4		40	4
	.2		.2	4		80	3
	.4		.4	4		155	4
	.1		.1	37	4–20	46	4
	.2		.2	37	4–20	54	4
	.4		.4	37	4–20	130	3
E., normal ♂	.1	”	.1	4		31	4
	.2		.2	4		29	6
	.5		.5	4		105	6
	.1		.1	37	4	34	4
	.2		.2	37	4	82	4
	.5		.5	37	4	148	4
H., hypopituitarism	.05	”	.05	4		34	4
	.1		.1	4		64	6
	.2		.2	4		132	4
	.5		.5	4		145	4
R., hypergonadotrophic hypogonadism	.3	”	.1	4		71	4
	.75		.25	4		116	4
	1.5		.5	4		140	4

TABLE III. Augmentation of Chorionic Gonadotrophin by Various Substances and Procedures.

Procedure	"Augmentor"	ml	Internat. units chorionic gonadotrophin ("A. P. L.")	Assay rats	
				Uterine wt, mg	No. of rats
Mix and store at 4°C	Polyvinylpyrollidone 25% in water	.2	.1	30	4
		.2	.2	65	4
		.2	.5	150	4
” ” ” ” ”	Raw egg white	.4	.2	57	4
		1.0	.5	102	4
” ” ” ” ”	Rat serum	.5	.5	72	6
		2.0	.5	138	4
Human plasma boiled 5 min., coagulum broken, mixed with hormone, stored at 4°C	Boiled human plasma (patient H.)	.5	.5	115	4
Plasma and hormone inj. separate subcut. sites	Human plasma (subject P.)	.5	.5	26	4
Assay rats castrated 3 days prior to inj.	” ”	1.0	5.0	33	4

indicator of pituitary gonadotrophin activity, ovarian weight is an insensitive indicator for measuring chorionic gonadotrophin and even with large doses, increases in ovarian weight are not striking. In some studies, such large amounts of chorionic gonadotrophin were used that almost maximal ovarian weight increases were produced, even without the addition of augmenting materials. Using a vaginal smear technic of assay, Pedersen-Bjergaard and Tønneson(9) have recently observed that the addition of colloidal materials to chorionic gonadotrophin would enhance its activity to the extent that a single injection would produce the effect of multiple injections of an aqueous solution of hormone. Thus, the choice of endpoint may account for failures to observe augmentation. Another important factor in interpreting studies of the augmentation phenomenon is the purity of the hormone preparations employed. With impure extracts, sufficient non-hormonal materials may be present to produce maximal augmentation and further enhancement of activity would not result from adding more "augmentor."

Since augmentation did not occur when plasma and chorionic gonadotrophin were kept separate and administered at different sites, the mechanism of augmentation is probably the same as that advanced for pituitary gonadotrophins, *i.e.* prolongation of absorption from the site of injection. Two other mechanisms might conceivably account for the enhancement of chorionic gonadotrophin activity by plasma: a synergistic action by minute amounts of endogenous gonadotrophin in plasma, and protection against inactivation (10). The first possibility seems unlikely since similar augmentation was observed with plasma from individuals having high, low, and normal endogenous gonadotrophin levels. Protection against inactivation would not seem to be an important factor, since aqueous solutions of the hormone are relatively stable, even when kept at 37°C.

Taking into account the phenomenon of augmentation, very small amounts of chorionic gonadotrophin can be detected in plasma. Considering that 0.2 unit of hormone mixed in plasma can produce a definite increase in uterine weight and that as much as 6.0 ml of plasma can be administered to the assay rats over a three-day period, it is possible to detect 0.03 units of chorionic gonadotrophin per ml of plasma. This is sufficiently sensitive to measure plasma levels of chorionic gonadotrophin in patients receiving therapeutic doses of the hormone(1).

Summary and conclusions. A 5- to 10-fold increase in the activity of chorionic gonadotrophin, as measured in rats, was produced by the *in vitro* addition of unaltered human plasma. Similar augmentation was observed when the hormone was mixed with boiled human plasma, rat serum, egg white and polyvinylpyrollidone. The augmenting abilities of plasma obtained from 2 hypogonadal individuals, the one having high, the other having low endogenous gonadotrophin levels, was no different than that of plasma obtained from normal individuals. Enhancement of hormonal activity did not occur when "augmentor" and hormone were kept separate and injected at different subcutaneous sites. It is concluded that the most likely mechanism of augmentation is prolongation of absorption from the site of injection.

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Received September 24, 1953. P.S.E.B.M., 1953, v84.

Evidence for Progestogen Secretion by ACTH-Stimulated Adrenals.* (20644)

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One method for testing the luteotropic potency of lactogenic or mammotropic hormone (MH) has been to stimulate the luteal tissue of hypophysectomized rats to form progestogen, which in turn induces a deciduoma to form in areas of endometrial trauma(1). Such a test has also been used in detecting luteotropic activity in rat placenta(2,3). However, in light of the very extensive studies on the many different steroids including progestogens that may be formed by the adrenal cortex(4,5) it has become necessary to ascertain whether or not adrenocorticotropic hormone (ACTH), by way of progestogenic adrenal steroids, might be responsible for deciduoma formation.

In earlier studies(1) the precaution of removing the adrenals had been taken in proving a luteotropic function for MH. It is now possible to report that ACTH preparations highly potent as adrenocortical growth stimulants are responsible for progestogen formation in the absence of the ovaries.

Procedure. The test animals were immature female Long-Evans rats hypophysectomized at approximately 30 days of age and injected for one week thereafter.† For luteotropic assays, it has been necessary to inject the rats prior to hypophysectomy with an ovulatory or luteinizing dose of pituitary or chorionic gonadotropins(1). A single injection of pregnant mare serum gonadotropin (PMSG) 4 days before hypophysectomy has served this purpose. Luteal tissue so induced shows functional cell hypertrophy after hypophysectomy only when stimulated with MH or rat placental extracts. Even when large masses of luteal tissue were induced in these immature ovaries they were incapable of secreting progestogen in the absence of the hypophysis or the hormones with luteotropic potency. The fractions to be tested for luteotropic activity were injected for 7 days beginning on the day of hypophysectomy. On the fourth day of injection a thread was inserted in one uterine horn; and necropsy was performed on the day following the last injection. In testing for ACTH-induced progestogen, neither pre-treatment with PMSG nor the presence of the ovaries was found to be necessary. The futility of attempting to gain even the slightest hint of the quality or quantity of progestogens formed by ACTH-stimulated adrenals under these circumstances was fully appreciated. Nevertheless, 2 available progestogenic steroids, progesterone and desoxycorticosterone acetate (DOCA) were tested for deciduoma-inducing potency. The pituitary, ovaries and adrenals may all be dispensed with when testing these substances.

Results and discussion. Table I shows the results obtained in a series of rats, injected with highly purified preparations of beef growth hormone (somatotropin), and sheep ACTH and MH. None of the controls injected with the ACTH vehicle (5% beeswax in peanut oil), and none of the somatotropin-treated animals showed deciduoma formation. This was true in both groups even when heavily luteinized ovaries had developed in response to PMSG prior to hypophysectomy. The somatotropin was estimated to have contained about 1% ACTH on the basis of adrenal histology. The MH contained less than an estimated 1% of ACTH; and activated the induced luteal tissue in daily subcutaneous doses of 100 γ (aqueous), or 50 γ in beeswax. As in an earlier series(1) the adrenals could be removed on the day of thread-insertion without interfering with MH-induced deciduoma formation. The ACTH preparations

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* This research was supported in part by grants from the Research Board of the University of California, the U. S. Public Health Service and Eli Lilly Laboratories.

† This test animal may be employed in assaying the 6 well known anterior lobe hormones, using histologic criteria in the thyroid, adrenal, ovary and tibia for their detection.

TABLE I. Deciduoma Formation and Adrenal Weights in Immature Hypophysectomized Rats Injected with Various Hormones.

Hormone	Daily dose, mg	No. of rats	Positive deciduoma	Avg wt l. adrenal, mg
None		27	0	4.3
MH-1	1.0	10	10	4.6
	.2	3	3	4.0
	.1	5	5	—
*	.05	3	2	4.4
ACTH-1	.060	3	3	19.4
”	.045	12	5	20.2
”	.03	3	0	15.5
2	.06	3	3	35.0
”	.06	3	0	—†
”	.03	7	3	21.2
3	.01	3	0	6.3
”	.03	3	3	22.5
4	.03	3	0	15.3
5	.03	3	0	12.5
6	.20	4	0	11.7
Somatotropin	.45	7	0	7.5
	.10	5	0	6.2
	.02	4	0	4.8
Progesterone	2.0	5	5	—
	1.0	3	3	—
DOCA	2.5	4	4	—
	1.0	3	1	—
Hydrocortisone	5.0	3	0	—

Notes: (1) All of the somatotropin and MH animals had been pretreated with PMSG. (2) All of the ACTH and one MH preparation (marked *) were inj. in 5% beeswax in peanut oil. (3) Avg wt of left adrenal in 12 normal rats, 32-36 days old, was 12 mg. † Adrenalectomized.

represent different fractions prepared by methods to be described elsewhere(6). Briefly ACTH-2 and -3 were obtained at a more advanced step in the purification procedure than ACTH-1. They were slightly more potent in adrenal weight increasing activity than ACTH-1; and had been subjected to peptic digestion, a procedure that destroys MH. ACTH-6 had been treated in such a way as to preserve a good ascorbic-acid depleting potency equal to that of ACTH-1, and to render it a relatively weak adrenal weight increasing preparation (Table I).

It was observed that ACTH usually induced deciduoma formation in those rats in which the weight of the adrenal had increased several fold over that of the average hypophysectomized control figure of 4.3 mg. However, some animals with the weight of the left adrenal as low at 15.8 mg showed deciduomata; whereas some with a weight as high as 23 mg did not.

In the greatly enlarged adrenals the sinusoids showed marked congestion with disruption of the reticuloendothelial lining and extravasation of blood in small scattered areas. The normally regular parenchymal pattern was greatly distorted by the rapid growth, vascular engorgement and necrosis. Some of the degenerated cells contained nuclear remnants, sudanophilic lipoids and birefringent material that was yellowish, not pink, in Sudan-stained sections. It appeared as though the adrenocortical parenchyma had become in part a holendocrine gland liberating whole cells and products of degenerated cells into the blood stream. Some normal-appearing cortical cells were seen also in the sinusoids of the medulla.

The abnormal appearance of some of the over-stimulated adrenals suggested that ACTH may have promoted the liberation into the blood stream of an unusual assortment and quantity of cholesterol derivatives. However, the probability remains that this might also be possible in a moderately stimulated, but nonpathological adrenal.

Deciduoma formation in the ACTH-treated rats was always associated with practically complete atrophy of the thymus and an average body weight loss of one g per day. The thymus could only be detected as a minute lymphoid remnant by staining the surrounding fat tissue in toto in alum carmine and clearing with methyl salicylate. The thymolytic effect is interpreted as being due to corticoids such as cortisone. Progesterone and DOCA were not found to be potent thymolytic agents; nor did they cause body weight loss such as has been observed with cortisone and hydrocortisone.

As shown in Table I both DOCA and progesterone induced deciduoma formation in the hypophysectomized immature rat when given alone and without pre-treatment with estrogen, in daily doses of one mg. This could probably be considered a borderline progestogenic dose of DOCA; but Astwood (7) has shown that as little as 0.1 mg (optimum 0.75 mg) of progesterone per day may induce deciduomata in pseudo-pregnant adult rats oophorectomized, but not hypophysectomized. Cortisone and hydrocortisone have never been considered progestogenic, and in this experiment the latter proved inactive in deciduoma tests when given as the acetate at the daily dose of 5 mg.

Pending identification and quantitation of the steroids formed in the ovary under the influence of MH, and in the adrenal following injections of ACTH by chromatographic technics(4,5,8,9) it may be a reasonable assumption that in equivalents of DOCA the daily effective secretion would be in the neighborhood of one mg—and of progesterone, somewhat less. However, it would seem more important to emphasize the fact that many steroids with different androgenic, estrogenic, progestogenic, etc., potencies may be formed in ACTH-stimulated adrenals (4,5,10,11), and that therefore the formation of a deciduoma would depend not only upon the total amount of progestogens secreted, but also upon the amounts of other steroids formed, *e.g.*, estrogens which, in optimal doses, enhance this action of progestogen and in high doses inhibit it(7,12,13).

Conclusions. 1. Daily doses of 30 γ of an ACTH suspension in beeswax and peanut oil injected for a week stimulated the adrenals of immature hypophysectomized and oophorectomized rats to form progestogen(s) as shown by the deciduoma test. 2. MH in daily doses as low as 50 γ accomplished this effect in immature hypophysectomized-adrenalectomized rats, the ovaries of which had previously been luteinized with PMSG. 3. Progesterone and DOCA in 7 daily doses of one mg induced deciduoma formation in immature hypophysectomized rats; hydrocortisone in 7 daily doses of 5 mg did not. 4. Since both MH and ACTH induce progestogen secretion, an assayist should study the respective target tissue of each (corpus luteum or adrenal cortex) for histologic evidence of activation rather than use biologic or chemical evidence of progestogen formation. An alternative would be to test for MH progestogen in hypophysectomized-adrenalectomized animals, and for ACTH-progestogen after hypophysectomy and oophorectomy.

We also wish to acknowledge generous supplies of progesterone from Parke, Davis and Co.; desoxycorticosterone acetate from Ciba Inc., hydrocortisone acetate from Merck and Co.; pregnant mare serum gonadotropin from Cutter Laboratory.

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Received September 28, 1953. P.S.E.B.M., 1953, v84.

Use of Ethylene Diamine Tetra Acetic Acid in Hypercalcemic Patients.* (20645)

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(Introduced by Alfred Gellhorn.)

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Increase in serum calcium concentration not uncommonly accompanies extensive osteolytic metastases. Such hypercalcemia represents inadequate renal excretion of the large amounts of calcium mobilized during rapid bone destruction(1). Compromise of renal function may occur from calcium precipitation in kidney parenchyma or dysfunction without appreciable histologic abnormality; difficulties in calcium excretion may thus be intensified. Efforts to modify hypercalcemia have not been highly effective. Infusion of citrate solutions has not fulfilled the clinical requirement, and decalcification of phlebotomized blood with an ion exchange resin and retransfusion of the hypocalcemic blood has proved cumbersome and inefficacious in our hands(2). Ethylene diamine tetra acetic (EDTA) and its sodium salts (sodium-EDTA) are capable of chelating many metallic ions. The calcium-EDTA complex at pH 7.4 is almost entirely nonionized and its calcium is non-precipitable by oxalate(3). Accordingly, the calcium atom of the calcium-EDTA complex is physiologically unavailable. Exchange does occur with endogenous calcium(4) and with metals more avidly chelated than calcium(5), but otherwise it is biologically inert. The EDTA molecule itself is metabolically unchanged *in vivo,* and acute toxicity of calcium-EDTA is remarkably low in many different species(6). Spencer and her collaborators(7) have administered 4 g of sodium-EDTA in solution to man over periods of 6 hours, without lowering oxalate precipitable calcium (hereinafter referred to as serum calcium): indeed total calcium per 100 ml was increased by an increment of circulating calcium-EDTA complex. Large amounts of chelated calcium, from 45% to 72% of the stoichiometric equivalent of infused EDTA, were excreted in the urine. In rabbits(8,9) and other laboratory animals(10) when sufficient quantities of parenteral sodium-EDTA are absorbed at a suitably rapid rate, decrease in serum calcium results, leading to asymptomatic hypocalcemia, tetany, or death. Sodium-EDTA has been administered intravenously to 5 patients with far-advanced cancer in whom widespread osteolytic lesions were associated with clinically significant hypercalcemia. Although clinical improvement was not seen, chemical, physiologic and pathologic data associated with the induced hypocalcemic state are of interest, and are herein presented.

Methods. Disodium-EDTA,‡ recrystallized 3 times, was dissolved in distilled water to make a 10% solution. Alkalinization to pH 7.4 was accomplished with 1 N NaOH. After autoclaving and immediately prior to use this was diluted to approximately 3% with 5% dextrose in water. Serum calcium was determined by precipitation with oxalate and oxidation by $KMnO_4$(11) or by hexanitratocerate in excess, employing back titration with ferrous ammonium sulfate to an orthophenanthroline endpoint(12). Calcium bound to EDTA was determined by ashing with concentrated H_2SO_4 at 600°C, followed by oxalate precipitation.

Case 1. 58-year-old female had unoperated breast cancer with extensive osteolytic metastases, symptomatic for 3 weeks. On admission, Mar. 17, 1952, she was anorexic, constipated, lethargic and apathetic. Tendon reflexes were normal, serum calcium 19.0 mg %, phosphorous (P.) 3.8 mg %, alkaline phosphatase (alk. p'tase.) 6.1 Bodansky units (Bod. u.) and NPN 82 mg %. With hydra-

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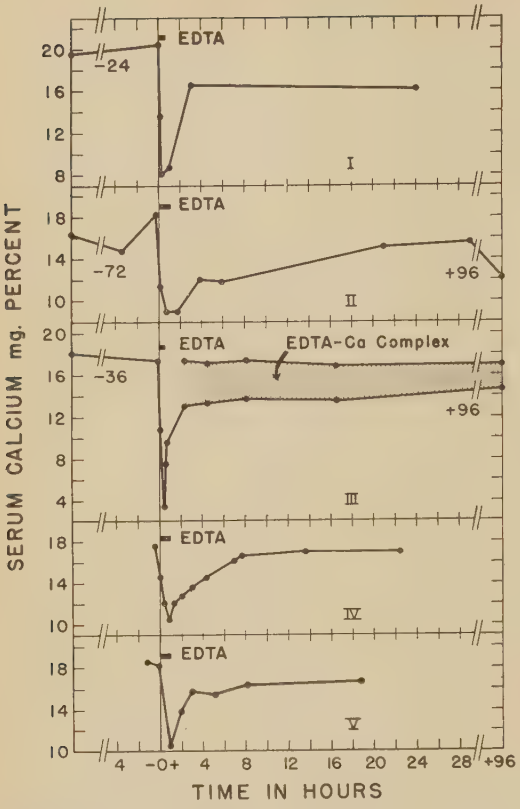
* These studies were supported in part by an institutional grant from the American Cancer Society.

† Medical Officer, National Cancer Institute, Bethesda, Md.

‡ The EDTA used in this investigation was kindly furnished by the Alrose Chemical Co., Providence, R. I., and the Bersworth Chemical Co., Framingham, Mass.



GRAPHS I-V. Response of serum calcium concentration to intravenous administration of sodium ethylene diamine tetra acetic acid.

tion NPN fell to 43 mg % but calcium level unchanged. Urinalysis showed 1+ proteinuria and rare white cells. Administration of EDTA initiated March 25, 25 mg given. Subsequently increasing amounts to 6 g in 45 minutes injected without change in calcium level. Clinical deterioration progressed. On April 10, 400 mg/kg body weight was given. At 15 minutes when approximately 20 g sodium-EDTA had been given (333/mg/kg body weight) she roused from stupor, complained of urge to defecate, of headache, and became restless. Chvostek's sign was negative and there was no hyperreflexia. EDTA was stopped and acute symptoms subsided, with return to obtunded state within one hour. Hypocalcemia occurred, with rapid rebound. (Graph I). Thirty-eight hours after EDTA, while on penicillin, patient expired with hyperpyrexia thought to be due to hypostatic pneumonia. No autopsy was performed.

Case 2. This 49-year-old female had disseminated carcinoma of breast involving soft tissue and causing universal osteolytic and osteoblastic skeletal metastases. On May 7, calcium was 11.4 mg %, P. 4 mg %, and alk. p'tase. 8.3 Bod. u. Admitted May 15, and following day calcium 17.2 mg %, P. 6 mg %, alk. p'tase. 5.8 Bod. u. Urine contained 1+ protein and occasional red and white cells. From May 20 through June 3 she received cortisone .3 g daily by mouth, without appreciable effect on serum calcium levels. NPN 104 mg %. On June 2, because of lethargy, weakness, constipation, and progressive uremia, 15 g of EDTA (approximately 225 mg/kg) were infused in 70 minutes. During infusion no objective changes were noted in sensorium, reflexes or vital signs, and patient did not complain. Serum P. levels remained between 5.2 and 5.6 mg %; changes in serum calcium shown in Graph II. On June 4, dihydrotestosterone 0.1 g daily I.M. begun. Ca. and P. levels returned to normal, alk. p'tase. increased suggesting new bone formation, and uremia subsided. In remaining 3½ months of life neither hypercalcemia nor azotemia recurred. On day of EDTA administration patient's blood count was, as usual, indicative of severe myelophthisic anemia with immature neutrophiles and nucleated red cells in peripheral blood. Platelet count was 120,000 though no previous values were recorded. Without other significant changes in formed elements, on days 4, 7, 9, 14, 18, 25, and 78 after EDTA respectively, thrombocytes (in thousands) were 44, 28, 34, 66, 78, 86, and 250. No changes in bleeding, venous clotting or prothrombin time appeared; epistaxes did occur, however, which were locally controlled. The relation of thrombocytopenia to EDTA, or remission, concomitant with administration of androgen is uncertain. Higher levels of red cells and hemoglobin, without changes in white cells, also occurred coincident with the platelet rise. Demise resulted from pulmonary insufficiency, pathologically attributable to abundant alveolar wall calcification. Metastatic calcification in kidney, although present, was remarkably less extensive. Generalized carcinomatosis was present, though inconspicuous in the lungs.

Case 3. This 49-year-old female had extensive bone destruction from multiple myeloma of 4 years' duration. She remained semi-ambulatory until admission on January 15, 1953, when calcium was 12.2 mg %, P. 3.1 mg %, alk. p'tase. 2.0 Bod. u. and NPN 43 mg %. Her urine findings were: sp. gr. 1.005, acid, protein 4+, and 1-3 white cells/hpf. She remained in bed because of pain, and was treated with 6-mercaptopurine. Increasing serum calcium level was detected during this period which by Feb. 4 was 18.2 mg %. Urinary calcium excretion ranged from 111 to 147 mg per 24 hours. She was lethargic and vomited frequently. 13.5 g of sodium-EDTA (approx. 160 mg/kg) administered intravenously in 29 minutes on Feb. 5. At approximately 25 minutes patient expressed apprehension of death and complained of urge to defecate. Obstructed inspiration and trismus appeared. EDTA was stopped at approximately 29 minutes; respirations ceased and cardiac sounds were not heard. Unsuccessful cardiac puncture seemed to stimulate one respiratory grunt. With a longer needle right ventricular blood was obtained and one cc epinephrine 1:1000 injected. Period of apnea is estimated at 3 minutes, and she regained consciousness in 20 minutes. The ventricular serum calcium was 3.4 mg %. Later venous levels shown in Graph III. In next 16 hours she voided 250 cc, but thereafter daily urinary output was less than 100 cc. Approximately 2.5 mg % of calcium-EDTA was detectable in serum even 96 hours after administration, attributed to the profound oliguria. On fourth day after EDTA, in the 55 cc urine voided, virtually all calcium excreted was complexed with EDTA. In spite of conservative management, patient died on Feb. 11 with acute renal insufficiency, and with progressive leucopenia due to 6-mercaptopurine. No autopsy performed.

Case 4. This 49-year-old female had had symptomatic multiple myeloma for 4 months, with extensive bony involvement, soft tissue tumor of thoracic wall, and proteinuria. One week earlier serum calcium elsewhere was 14.5 mg % and BUN 15 mg %. On admission Apr. 6, the urine showed heavy proteinuria and serum calcium 17.6 mg %, P. 5.2 mg %, alk. p'tase. 2.3 Bod. u., and NPN 67 mg %. In spite of adequate hydration and supportive therapy, on Apr. 15 serum calcium was 20.2 mg % and NPN 171 mg %. On Apr. 16, 10 g sodium-EDTA (approx. 175 mg/kg) given intravenously in 54 minutes. No lessening of torpor nor other subjective reaction occurred. Calcium values are shown in Graph IV. In the remaining 4 days of life there was no alteration in course of her disease. She died Apr. 20 from progressive renal insufficiency. At necropsy, universal multiple myeloma with marked osteolysis was found, and the kidneys showed mild Bence-Jones protein nephrosis. In addition, many tubular cells showed marked vacuolization reminiscent of sucrose nephrosis. Minimal metastatic calcification was present. The marked hydropic degeneration was entirely similar to that found in patient No. 5 (v.i.) and is felt to be independent of other renal pathology present. These changes may have been produced by sodium-EDTA; they are not those recognized as associated with myeloma, amyloid, or hypercalcemia (Fig. 1).

Case 5. 32-year-old female had had malignant melanoma of the rectum for one year despite attempted removal. Inguinal node metastases and extensive destruction of the bony pelvis and lumbar spine had occurred, and she was first admitted for bone pain. Serum calcium was 10.6 mg % on Mar. 30, and on Apr. 21 NPN was 30 mg %. By Apr. 27 calcium was 19.2 mg %, P. 5.4 mg %, alk. p'tase 5.6 Bod. u., and NPN 60 mg %. Urine revealed only 1+ protein. Her condition deteriorated markedly and she became semicomatose. On May 7, 9.5 g of sodium-EDTA (approx. 235 mg/kg) administered intravenously in 60 minutes. Serum calcium dropped as seen in Graph V. In the 4 days until death, hypercalcemia continued and uremia progressed. At autopsy widespread malignant melanoma without ureteral obstruction and intense vacuolization of tubular epithelium were found. (Fig. 2). Metastatic calcification was present in some tubular cells. Intracellular eosinophilic masses, in close juxtaposition to calcified areas were also seen and raise the possibility that cal-

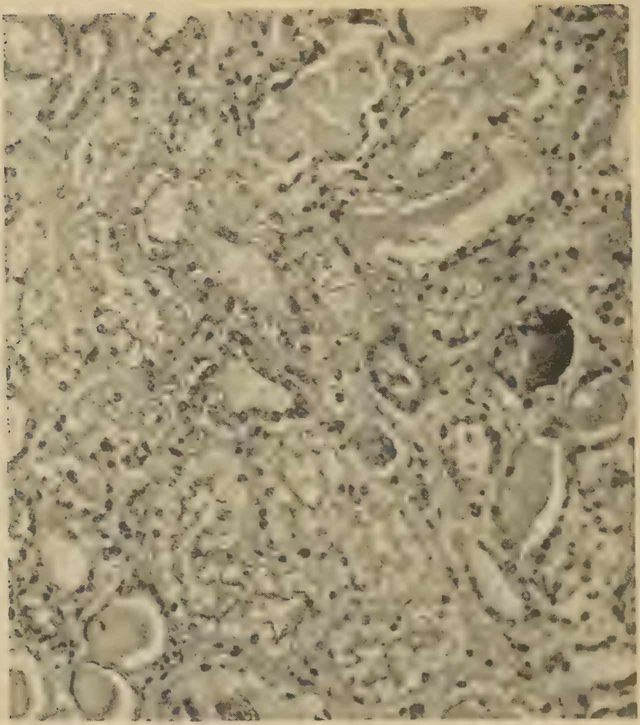


FIG. 1. Hydropic degeneration of renal tubular epithelium in patient #4. Tubules distended with Bence-Jones protein and metastatic calcification also shown.

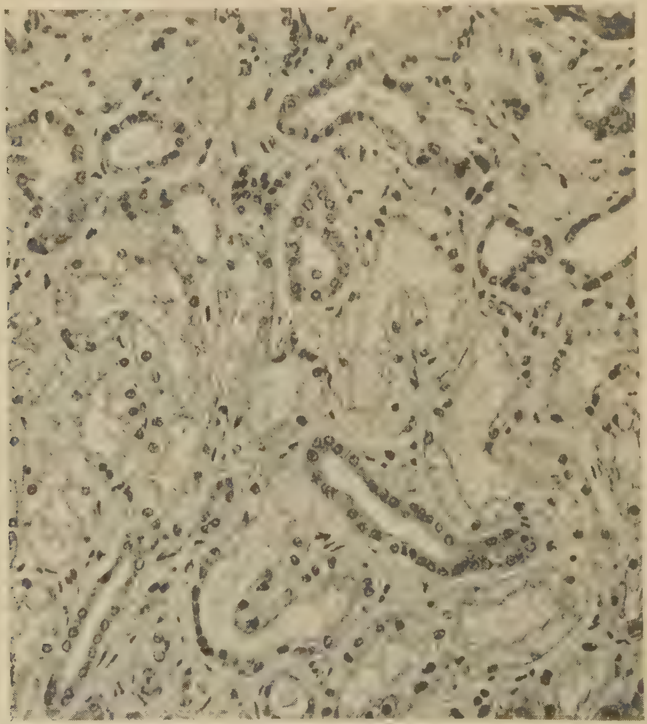


FIG. 2. Vacuolization of tubular epithelium in patient #5. Relatively normal and calcific tubules also shown.

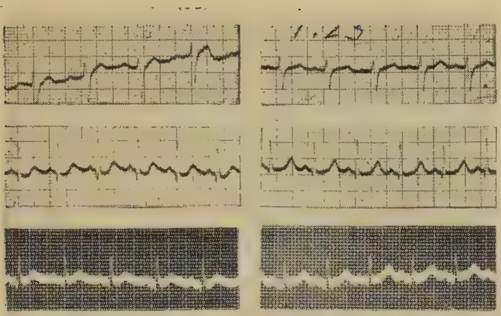


FIG. 3. Electrocardiograms of patients #3, #4 and #5 (top to bottom) during hypercalcemia (left) and normocalcemia (right). Consult Table I. Normocalcemic record in patient #3 taken before apnea. See text.

TABLE I. Cardiac Repolarization at Varying Levels of Serum Calcium.

Bazzett's formula $K = \frac{Q\text{-}T}{\sqrt{R\text{-}R}}$.

Patient	Time, min.	Ca, mg %	Q-T, sec.	R-R, sec.	K
#3	0	17.4	.30	.67	.37
	15	10.8	.38	.60	.49
#4	0	17.5	.35	.50	.49
	77	10.5	.38	.56	.51
#5	0	18.2	.32	.53	.44
	67	10.5	.33	.49	.47

cium may have been present in these loci before the sodium-EDTA infusion. There was a small amount of metastatic calcification in the lungs, but none in other tissues.

Discussion. The rapidity with which serum calcium levels can be depressed with EDTA at dosage levels employed in this study is noteworthy. As in experimental animals, this is a function not only of amount administered but of rate. There is no reason to assume that homeostasis of serum levels during slow infusion, or elevation toward normal following rapid infusion is a function of dissociation of calcium-EDTA or of renal reabsorption of unbound calcium. Rather, as Spencer and her collaborators(7) indicate, in the presence of excess calcinuria, this homeostasis must represent mobilization of calcium from bone. The speed with which this calcium shift occurs, and its control without apparent overshoot are of interest. The role of the parathyroids in this phenomenon cannot be deduced from data presented, nor is the reason for return to an unphysiologic level elucidated.

Electrocardiograms were taken within one minute of blood specimens during experimental periods in patients No. 3, No. 4, and No. 5. The K in Bazzett's formula $K = \frac{Q\text{–}T}{\sqrt{R\text{–}R}}$ rose slightly in each instance from the control to the depressed calcium level. This is in accord with the generally accepted slowed repolarization during hypocalcemia (Fig. 3, Table I). Initially high K values, the presence of uremia, and the near-moribund state allow little significance to be placed in the small variations observed.

The death of patient No. 1 was probably independent of this study. The possibility that thrombocytopenia in patient No. 2 may have been related to the compound cannot be summarily dismissed, although similar reports have not been found. The excessive hypocalcemia achieved in patient No. 3 resulted in a series of events terminating in death. In patients No. 4 and No. 5 the peculiar hydropic degeneration of tubular epithelium found at autopsy, in each instance 4 days after the administration of EDTA, may represent an effect of this agent. In neither instance was this lesion recognizable clinically, nor did it seem to influence the patients' survival. Similar findings have been described after administration of calcium-EDTA to dogs, but untreated and saline-treated control animals were also found to have vacuolization of their renal epithelium(13). The role of EDTA in causation of the lesion in the experimental group was therefore discounted. In rare cases tubular vacuolization has also been observed at autopsy in patients with cancer who have not received EDTA(14,15). The surprisingly little renal calcification in patient No. 2 contrasted to the abundant pulmonary calcification present, admits of the possibility that renal calcification may in some way have been modified by EDTA presented to the kidney for excretion.

Failure to improve the condition of 5 patients with cancer, bone destruction and hypercalcemia by transient depression of serum calcium is not wholly unexpected. In no instance did azotemia regress, nor did the torpor often ascribed to hypercalcemia remit during the sodium-EDTA infusion except evanescently in patient No. 1. The eventual return of hypercalcemic levels to normal in patient No. 2 is not attributed to EDTA. With the exception of patient No. 3, no apparent modification of survival was effected by the procedure.

Summary. 1. A sodium salt of ethylene diamine tetra acetic acid at pH 7.4 has been administered rapidly intravenously to 5 patients with hypercalcemia from bone destruction. 2. Decrease in serum calcium concentration has been produced but this is of remarkably short duration and unassociated with clinical benefits. 3. Two instances of renal tubular vacuolization following administration of sodium-EDTA have been observed.

The authors are indebted to Dr. Edith E. Sproul for her interpretation of pathologic material. They also wish to express their appreciation to Dr. Jane V. Gang for assistance; and to Drs. M. Garcia, L. McDaniel, J. Nork and C. O'Sullivan, who, as medical students, participated actively in this investigation.

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Received July 22, 1953. P.S.E.B.M., 1953, v84.

Effect of Electroshock Therapy on Blood Lactic Acid and Pyruvic Acid. (20646)

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Electroshock therapy (EST) is widely used in the treatment of certain mental disorders. However, the biochemical changes in blood constituents resulting from such traumatic electrical stresses to the body are little known. Our immediate interest in this problem was centered around the changes in lactic acid and pyruvic acid levels appearing in the blood after the extreme muscular excitation induced by EST. The evidence available in the literature concerning the effect of EST on the lactic acid and pyruvic acid levels, as well as on other constituents, in the blood is both fragmentary and inconclusive(1-3). In most cases reported, the time intervals of blood sampling of patients receiving EST were chosen arbitrarily and consequently have given rise to conflicting and unsystematic data. We undertook this investigation to ascertain precisely the changes occurring in both lactic acid and pyruvic acid levels of the blood following EST.

Methods. Ambulatory patients, ranging from 20 to 40 years of age and carrying a diagnosis of chronic schizophrenia, were employed in this study. Patients having a relatively quiet awakening after EST were

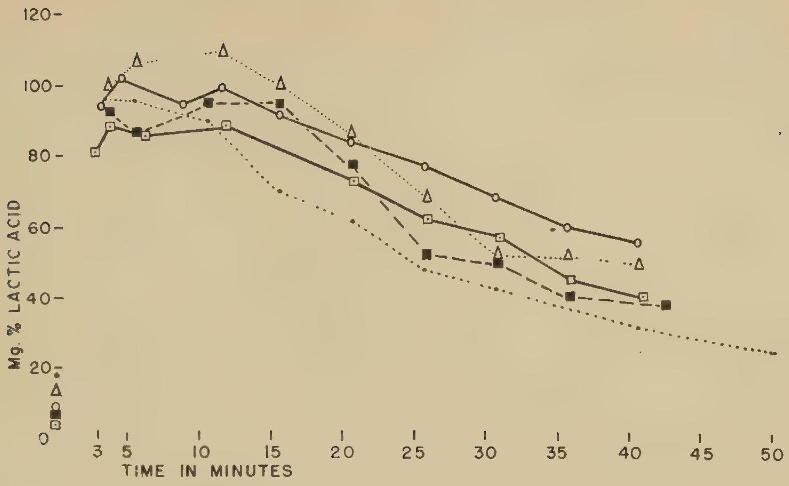

FIG. 1. Various symbols represent lactic acid values of different patients. Pre-shock values of each patient are shown on the ordinate. Abscissa represents the time from onset of shock.

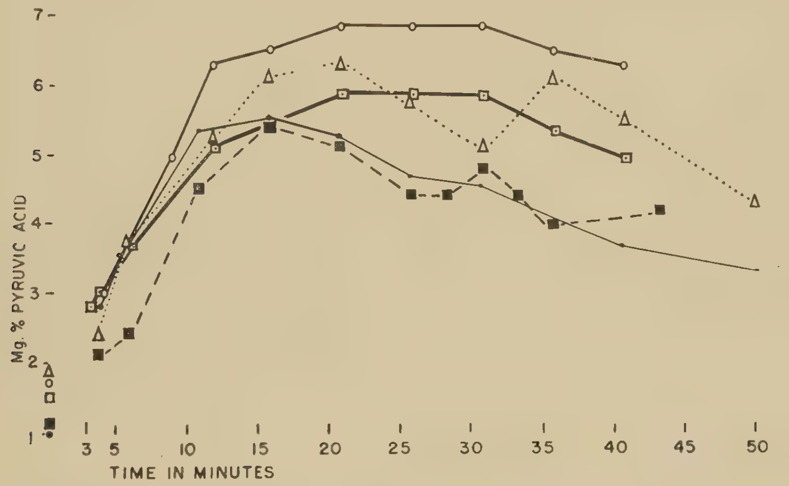

FIG. 2. Same as Fig. 1, except the values are for pyruvic acid.

selected. Each subject was given 3 to 6 grains of sodium amytal by mouth prior to shock. EST was administered by the "Electronicraft" machine with the initial current at 125 milliamperes and with automatic resistance compensation. Maximum current of 350 milliamperes was reached within 2½-3 seconds. The current was reduced to 250 milliamperes in 5 seconds then was gradually decreased depending on the response of the patient and was abruptly terminated during the later part of the clonic phase of the convulsion. The average length of the total treatment was 35 to 40 seconds. Samples of venous blood were collected without the aid of a tourniquet and immediately placed in cold 10% trichloroacetic acid. A pre-shock sample was removed and samples were taken following the clonic phase of the convulsion at approximately one-half minute intervals up to 3 minutes and every 5 minutes thereafter. The method used for the determination of lactic acid was that of Barker and Summerson(4). Lithium lactate, which is used by most workers as a standard for this method, appeared to pick up water and thus furnished only relative results. Consequently, we employed as our lactic acid standard a sample of non hygroscopic calcium L(+) lactate of known water content which was a mixture of hydrates: H_2O, 23.4%; ash

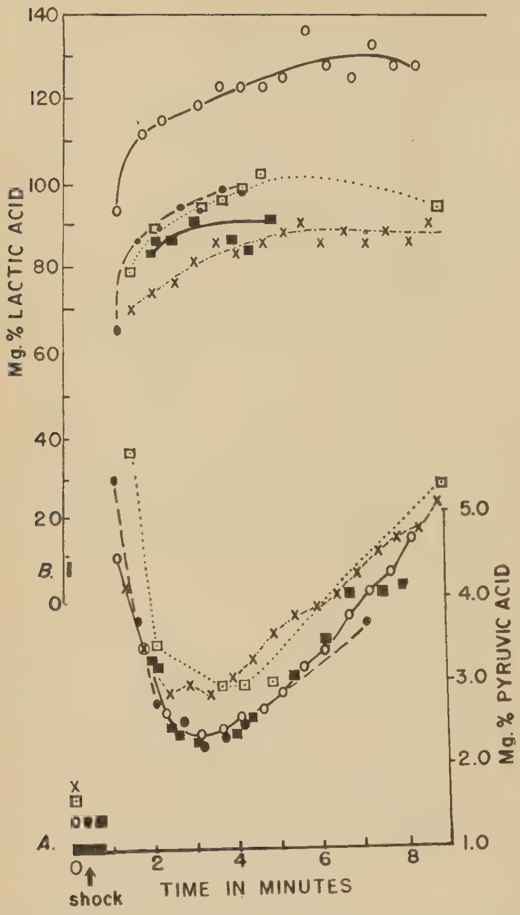

FIG. 3. Time scale (abscissa) is enlarged to show drop in blood pyruvic acid levels and corresponding rise of blood lactic levels. Various symbols in lower left corner (marked A) represent pre-shock levels of pyruvic acid. Bar on the left ordinate (marked B) represents variation in pre-shock levels of lactic acid.

($CaCO_3$) calculated, 35.5%, theory, 35.2%. The lactic acid determinations were made on the same day the blood samples were collected, since on standing the lactic acid concentration increased significantly. The method for determining pyruvic acid was that of Friedmann and Haugen(5) as modified by Fister(6). Samples for pyruvic acid determination were stored at 5°C for 24 hours in order to destroy interference by acetoacetic acid. The Friedmann and Haugen procedure(5) and the Lichtenbelt and Florijn method(7) (heating the fresh deproteinized blood filtrate in a boiling water bath for 5 minutes) gave similar results.

Results. The blood lactic acid level for each patient, following EST (Fig. 1), rose sharply to a peak within 3 to 5 minutes, then declined approaching the normal pre-shock level usually within 1½ hours. The concentrations at peak levels increased approximately 6-fold over pre-shock levels. The blood pyruvic acid reached a peak approximately three times the pre-shock level after about 20 minutes (Fig. 2).

When blood samples were drawn at 20 to 30 second intervals, it was observed that both the lactic acid and pyruvic acid concentrations rose immediately following EST. However, after thirty seconds the pyruvic acid level dropped, falling to a minimum at the same time that the lactic acid reached its maximum (Fig. 3). The variation among patients of these two metabolites, at any particular time after shock, was quite large. However, as shown in Fig. 4, the ratio of lactic acid to pyruvic acid (L/P), at any particular time, was quite constant. It seems clear that unless a comprehensive picture of the fluctuations of the levels of lactic acid and pyruvic acid (or any other metabolite) is obtained, an interpretation of data based on arbitarary spot-checking of the concentrations of these metabolites in the blood can only lead to confusing and conflicting results. Since during the first three minutes there was a drop in pyruvic acid levels with a corresponding rise in lactic acid levels,

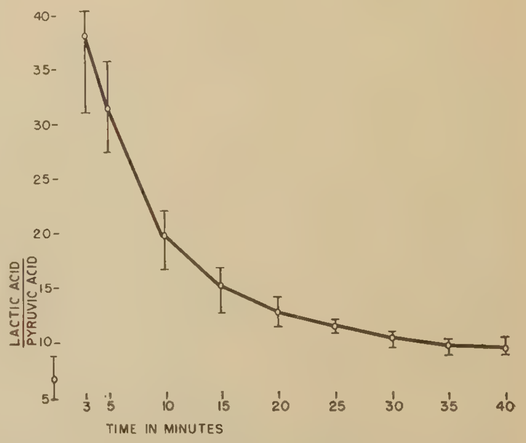

FIG. 4. Points (circles) represent mean ratio of lactic acid/pyruvic acid for same patients illustrated in Fig. 1 and 2. Bars represent largest deviation from the mean. Circle located on the ordinate represents mean ratio of pre-shock levels.

it would appear that the Embden-Meyerhof scheme represented the dominating event during this time interval. Also, since after the first 3 minutes, the keto acid levels increased as the hydroxy acid levels decreased, and since the concentrations of these metabolites then returned to normal pre-shock levels, it would appear that the aerobic phase predominated during this time interval. It is of considerable interest that the L/P ratio, at any particular time after shock, of all patients studied fell within a small range.

Further investigations are in progress to determine the relationship between the variations in the concentrations of lactic acid and pyruvic acid, or their ratios, as an index of the relative intensity of the convulsion in EST.

Preliminary data obtained on the blood levels of glucose, sodium, potassium, and calcium, indicated that the concentration of these metabolites varied considerably over a sixty minute period following EST, rising and falling several times during this time interval.

Summary. A simultaneous measurement has been made of lactic acid and pyruvic acid changes in the blood of neuropsychiatric patients before and after extreme muscular excitation induced by electroshock therapy (EST). The blood lactic acid level of all patients following EST rose sharply to a peak (six times pre-shock level) within 3 to 5 minutes and then returned to the normal level after 1 to 1½ hours. The blood pyruvic acid level of all patients rose immediately following EST for thirty seconds (4-6 mg %), then fell to a minimum (2-3 mg %) within 3 to 5 minutes and rose again to a peak three times the pre-shock level 20 min after EST.

We would like to thank Mr. Roscoe R. Schaffert and Miss Marion Amos for their kind assistance with the analyses and Dr. Seymore Bird for his kind cooperation in dealing with the patients. The calcium lactate was obtained through the courtesy of Dr. Merrill N. Camien, Department of Biochemistry, University of California at Los Angeles.

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Received August 25, 1953. P.S.E.B.M., 1953, v84.

Influence of Hibernation on Survival Time and Weight Loss of X-Irradiated Ground Squirrels.* (20647)

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Numerous studies on the influence of temperature on the radio-sensitivity of various biological systems have been conducted and it has been concluded(1) that temperature changes within the range which can be tolerated by most living systems do not directly modify radiation reactions. However, the tissue injury resulting from the interaction of the products of ionizing radiations with cellular constituents is delayed when the temperature of the organism is maintained below normal following irradiation and survival is thereby prolonged. Although this effect has been demonstrated in several types of cells and tissues by a number of investigators, its occurrence in hibernating mammals(2-4) is of particular interest because it provides another approach for studies on the mechanism responsible for the injurious action of ionizing radiations on mammalian tissues.

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* This study was supported by funds provided under contract with the USAF School of Aviation Medicine, Randolph Field, Texas.

For the application of hibernating animals to research in radiobiology it is desirable to use a species which can be readily obtained, easily maintained under laboratory conditions and in which hibernation can be induced throughout the year. We have found that the hibernating ground squirrel (*Citellus tridecemlineatus*) meets these requirements and thus serves ideally for investigating the influence of toxicants on a hibernating species. The present communication describes experiments on the susceptibility of hibernating and non-hibernating ground squirrels to lethal doses of x-irradiation. The results of this investigation have shown that animals irradiated while in hibernation exhibit a prolonged survival time provided they are maintained in the poikilothermic state following irradiation.

Materials and methods. Male and female ground squirrels were trapped in the vicinity of Chicago during the summer months by placing a wire cage over the entrance to the burrow and flooding the burrow with water, forcing the animals into the cage. They were maintained in an air-conditioned laboratory at 68-75°F in individual cages and fed Rockland Rat Diet supplemented biweekly with carrots and cabbage. Hibernation was induced in adult animals of this species by placing the animals in a cold room at 5°C for 24 to 48 hours without food or water. Hibernation in this species is more profound than in other available hibernating animals and it is possible to expose the hibernating ground squirrels to light, noise, and even certain surgical procedures without arousing the animals. Furthermore, the hibernating ground squirrel does not return spontaneously to the homeothermic state as frequently as do most other hibernating species and in this respect offers a distinct advantage for studies involving hibernation. X-irradiation was administered in a single whole-body exposure by means of a Keleket Therapy X-ray Machine (250 KVP, 15 ma, 0.25 mm copper and 1 mm aluminum added filtration, 75 cm target distance, dose rate 35-36 r per minute). Groups of 12 animals were irradiated simultaneously in a rotating aluminum cage placed perpendicularly to the central ray so that each individually caged animal received an identical dose. When animals were irradiated while in the hibernating state, the entire cage was enclosed in a refrigerated box maintained at 5°C during the exposure.

Results. To ascertain the susceptibility of ground squirrels to x-irradiation, 6 groups each containing 12 non-hibernating animals were exposed to doses of x-ray ranging from 400 r to 1200 r. Observations on the mortality and changes in body weight were made daily for 30 days following irradiation. The results of these measurements on 72 animals indicated that the approximate 30-day LD_{50} of x-ray for ground squirrels is 700 r. Non-hibernating ground squirrels exposed to 800 r of x-ray and kept at room temperature following irradiation exhibited 80% mortality within 10 days and 100% mortality within 30 days after irradiation. In a group of 24 ground squirrels, which were irradiated while in the hibernating state and maintained at 5°C thereafter, no mortality was observed within 30 days after the administration of 800 r. However, when 6 of these animals were returned to room temperature either at 2 weeks or at 4 weeks after irradiation, they developed the typical radiation syndrome and died within 10 days. Thus the ultimate mortality was the same as if the animals had been irradiated on the day that they were returned to room temperature and the homeothermic state. Similar results were obtained using doses of 1000 r and 1200 r of x-ray. In 2 groups of 12 non-hibernating ground squirrels given 1200 r, death of all of the animals occurred within 6 days following irradiation, whereas in the animals given this dose while in hibernation and maintained in hibernation following irradiation, no deaths occurred within 30 days after irradiation. However, when 6 of the hibernating, irradiated ground squirrels were returned to room temperature all of these animals died within 6 days.

When 12 ground squirrels were irradiated while in the non-hibernating or homeothermic state and then maintained at 5°C, none of the animals went into hibernation and these animals exhibited only a slight prolongation of survival time as compared with non-hibernating animals maintained at room temperature following irradiation. It is thus clear that maintaining the animals at a low environmental temperature following x-irradiation does not prolong the survival time in this species. In order to obtain the increased survival time following x-irradiation it is necessary that the animals be in hibernation during the irradiation and remain in hibernation following the exposure to x-ray. In the few instances in which the hibernating, irradiated ground squirrels spontaneously came out of hibernation after irradiation the animals did not return to the poikilothermic state and died within a few days even though they were maintained at 5°C. The prolonged survival time observed in the hibernating, irradiated ground squirrels did not alter the ultimate mortality of these animals, however, since even those animals in which hibernation was not spontaneously nor artificially terminated eventually died. Deaths began in these animals on the 54th day after 800 r, on the 42nd day after 1000 r, and on the 38th day after 1200 r of x-ray. It, therefore, appears that although the development of sufficient radiation injury to cause death of the animals is markedly delayed by hibernation, it is not prevented.

Measurements of the body weights of irradiated ground squirrels were used as an indication of the response of the hibernating and non-hibernating animals to various doses of x-ray. The results of body weight measurements following 800 r of x-ray are shown in Fig. 1, in which each value on the curves represents the average for at least 12 animals. From these data it may be seen that ground squirrels which were irradiated while in hibernation and maintained at 5°C thereafter lost about the same amount of weight as did non-irradiated hibernating controls. When these irradiated animals were returned to the homeothermic state by placing them at room temperature a rapid decrease in weight occurred terminating with death of the animals. Ground squirrels given 800 r while in the homeothermic state and maintained at room temperature following the irradiation exhibited a rapid decline in body weight. Thus the changes in body weight supported the mortality data and indicated that radiation damage is markedly retarded in irradiated, hibernating animals.

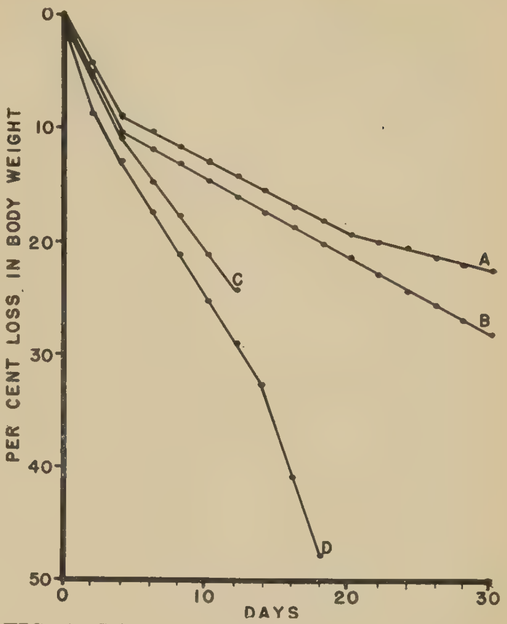

FIG. 1. Effects of hibernation and x-irradiation (800 r) on body wt of ground squirrels *(Citellus tridecemlineatus)*. A. Unirradiated, hibernating. B. Irradiated during hibernation and maintained thereafter at 5°C. C. Maintained in homeothermic state during and following irradiation. D. Irradiated while in the homeothermic state at room temperature and maintained at 5°C after irradiation.

Discussion. The initial chemical events such as the production of free radicals or the direct alteration of biological compounds by ionizing radiations are probably not influenced by temperature differences of the magnitude seen in hibernating and non-hibernating ground squirrels. Evidence in support of this statement was obtained in this investigation by the observation that although hibernation markedly prolonged the survival time of x-irradiated ground squirrels it did not influence the ultimate mortality of these animals. This finding was interpreted as meaning that the same initial events occurred in both the hibernating and non-hibernating animals on exposure to x-irradiation, but that in the hibernating animals the repercussions of this initial damage are not manifested to the same degree as in the normal animal. The marked lowering of the metabolic rate during hibernation is apparently responsible for the prolongation of the survival time in the irradiated, hibernating animals either through postponing the tissue damage or by markedly slowing the rate of disruption of the cellular metabolic processes by x-ray. Studies by Smith and Grenan(3) have indicated that at least some of the blood changes characteristically seen after x-ray, do not occur while irradiated woodchucks and marmots are in the hibernating state. Petersen and DuBois (6) found that lethal doses of x-ray produce an increase in the activity of alkaline phosphatases of the spleens of ground squirrels. The amount of increase in enzyme activity was less in hibernating, irradiated animals than in non-hibernating animals receiving the same dose of x-ray.

The results of this investigation showed that ground squirrels irradiated while in the homeothermic state and maintained thereafter at 5°C failed to exhibit any significant prolongation of survival time. It seems likely that the inability of irradiated ground squirrels to undergo hibernation with a consequent decrease in metabolic rate accounts for this lack of protection. It has been demonstrated that amphibians(5) kept at low temperatures after x-irradiation exhibit a prolonged survival time. Thus if it were possible to induce hibernation and the coincident lowering of the metabolic rate immediately following irradiation, a protective effect might be noted in ground squirrels irradiated while in the non-hibernating state. Extension of the results of this investigation may provide further insight into the mechanism of radiation lethality.

Summary. The LD_{50} of x-ray for non-hibernating ground squirrels (*Citellus tridecemlineatus*) is approximately 700 r. Non-hibernating animals given 800 r of x-ray exhibited over 80% mortality within 10 days. Ground squirrels irradiated while in hibernation and maintained in the hibernating state following irradiation exhibited no mortality within 30 days following exposure to x-ray, but when these animals were returned to the homeothermic state either at 2 weeks or at 4 weeks after irradiation, death ensued within 10 days. Animals which were maintained in hibernation following lethal doses of x-ray eventually succumbed, although survival was greatly prolonged. Thus, while hibernation markedly increased the survival time in this species following lethal doses of x-ray, it did not alter the ultimate mortality.

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Received August 28, 1953. P.S.E.B.M., 1953, v84.

A Growth Factor Required by *Donovania granulomatis*.*† (20648)

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Little is known concerning the exact requirement for growth of the Donovan body (*Donovania granulomatis*). Anderson(1) reported the cultivation of the Donovan body

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* This investigation was supported by grants from Lederle Laboratories Division of American Cyanamid Corp. and the Division of Research Grants of the National Institutes of Health, Public Health Service.

† A preliminary report was presented at the meeting of Fed. Am. Soc. for Exp. Biol., New York City, April 14-18, 1952.

in the yolk sac of the chick embryo, and later (2), on the embryonic yolk alone. Dunham and Rake(3) and Rake and Oskay(4) were able to grow the Donovan body on slants consisting of tryptose-beef heart infusion broth, Leventhal's stock broth and agar but did not obtain luxuriant growth. Dienst and associates(5) developed a medium containing fresh egg yolk, Bacto-peptone, Bacto-tryptone, dextrose, sea salt and agar in which luxuriant growth of the Donovan body was obtained. In a recent investigation of the bacteriological behavior of *D. granulomatis,* Goldberg, Weaver and Packer(6) studied the unknown factor found in fresh egg yolk required by the organism for growth. The factor varied in amount in eggs from different sources and seemed to be most abundant in eggs from chickens on a "natural diet," although it was also found in infertile duck, goose and turkey eggs. These investigators were able to extract with Locke's solution from chicken egg yolk (coagulated at 80-85°C for 10 minutes) an active material which gave a positive biuret and Molisch test and was not filterable through an ultrafilter. None of the following substances would replace egg yolk to support growth of the Donovan body: rabbit liver extract, grass extract, 1% yeast extract (Difco), rabbit liver extract with 1% gastric mucin, thiamin hydrochloride, cystine, asparagine, biotin, pimelic acid and a synthetic medium containing casamino acids, glucose, cystine, tryptophan, asparagine, uracil, adenine, guanine. xanthine, pantothenic acid, riboflavin, nicotinic acid, biotin, pyridoxine, folic acid, thiamin hydrochloride and p-aminobenzoic acid. On comparison of the morphology of the Anderson and Dienst strains of *D. granulomatis,* Goldberg and associates found differences in morphology resulted from differences in the media used by these investigators.

The present paper is concerned with a study of the factors found in egg yolk which are necessary for the growth of the Donovan body.

Experimental. In general, all procedures used in this study were repeated 3 or more times with consistent results. The eggs used were day-old eggs from a reliable source which supplied eggs with a high content of the growth factor. *Assay procedure.* The preparation to be tested is dissolved or suspended in sufficient fluid to make 100 ml and then is added in place of the diluted egg yolk to the medium of Dienst *et al.*(5).‡ The pH of the medium is adjusted to between 7.2 and 7.4 and the preparation is sterilized by autoclaving at 15 lb pressure for 15 minutes. Two of the tubes containing 5 ml each of the medium to be tested are inoculated with 1/10 ml from a 2-day-old culture of *D. granulomatis* and then are incubated for 2 days before being similarly transferred to new tubes of the same medium. At the time each transfer is made, the extent of growth is determined by examining a stained smear made from a loopful of the culture. If the organism will grow luxuriantly on the test medium through 4 or 5 transfers, this is considered to indicate an adequate level of the growth factor in the medium. The cultures of the Donovan body used in these studies were the A strain and Z strain isolated in 1947 from active lesions of patients suffering from granuloma venereum and have been subcultured since that time on the medium of Dienst and associates(5). The stock cultures are kept at incubator temperature and transferred 3 times per week. This assay procedure proved to be adequate to show gross differences in distribution and activity for various preparations and possible sources of the growth factor, although the procedure was modified at a later date to give results in terms of arbitrary "units of activity."

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‡ Bacto peptone 0.3 g, Bacto tryptone 0.3 g, glucose 0.3 g, sea salt or crude rock salt 0.2 g and agar 0.12 g. This is dissolved in 100 ml water and the pH is adjusted to 7.3. After autoclaving, the yolks from 2 fresh eggs are removed aseptically, diluted with 50 ml of sterile saline and added to the medium. The approximately 200 ml of medium resulting is then measured aseptically into culture tubes (5-6 ml to a tube). The tubes are incubated for 30 minutes in a water bath at 56°C after which they are ready for use. The portion of the medium other than the diluted yolk was for convenience termed "the basal medium" and the material to be tested was added to this in place of the egg yolk. If the volume of the substance to be tested was likely to be high, a double strength basal medium was prepared to allow for greater dilution by the test substance.

Heat stability. Growth was adequate on the Dienst medium which had been autoclaved at 20 lb for 30 minutes though this resulted in coagulation of the egg yolk proteins and thus changed the physical properties of the medium from that of the medium as originally used. This and other studies of heat stability indicated that autoclaving at 15 lb pressure for 15 minutes as done during the assay procedure did not result in a significant loss of the growth factor. This is in contrast to the results of Goldberg *et al.*(6) who observed a greater degree of thermal instability in the Dulaney medium (consisting of egg yolk and Locke's solution) then was noted with the more complex Dienst medium used in the present study.

Fractionation of egg yolk. a) Dialyzable constituents. Yolks from 2 eggs diluted with an equal volume of saline was dialyzed for 36 hours through a collodion membrane or cellophane against distilled water, saline or the basal medium. Neither the aqueous or saline dialysate added to the basal medium nor the basal medium dialyzed against the yolk supported growth. *b) A protein-free aqueous extract of egg yolk.* The yolks of 10 eggs were diluted with distilled water to make a volume of one liter. This was heated to boiling with constant stirring to coagulate the protein. On filtering with the aid of Filter-Cel, a clear filtrate was obtained which was concentrated to a small volume at a low temperature by vacuum distillation. In another similar experiment no Filter-Cel was used and suspended lipids were removed from the filtrate by extraction with petroleum ether. On assay, these protein-free, aqueous extracts of egg yolk failed to support growth of the Donovan body. *c) Protein and lipid fractions from egg yolk.* The yolks of 4 eggs were added with vigorous stirring to 200 ml of a mixture of 2 parts of alcohol with one part of ethyl ether at a temperature of 2°C. After twenty minutes the mixture was filtered and the precipitate was returned to the beaker. One hundred ml more of alcohol-ether mixture was added with stirring to the precipitate and after 15 minutes the mixture was again filtered. After 3 more such washings, the precipitate was placed in a vacuum desiccator over $CaCl_2$ to remove the last traces of ether. The alcohol-ether mixture used in the process was evaporated to dryness and the last traces of ether removed in a similar fashion, since small amounts of ethyl ether inhibit growth of the Donovan body. The lipid fraction failed to support growth of the Donovan body while the medium containing the precipitated protein fraction produced luxuriant growth. The dried protein fraction still retained the growth factor after 3 months at room temperature. After a year such a preparation was no longer active. Long continued extraction of the protein fraction in a Soxhlet extractor with successive portions of alcohol, ethyl ether, and petroleum ether did not effect the growth-promoting activity of the protein fraction. *d) Separation of yolk proteins.* Lipovitellin, vitellin and lipovitellinin were separated from egg yolk as described by Alderton and Fevold(7) and Fevold and Lausten(8), except that a Spinco preparative model ultracentrifuge was used rather than the Sharples super centrifuge. In the preparation of phosvitin the procedure of Mecham and Olcott(9) was used. The other proteins of the yolk were not isolated but were tested in fractions removed in the process of isolating the proteins mentioned above. The vitellin preparation contained the major proportion of the activity of the yolk. The other fractions either were inactive, or of comparatively low activity.

Effects of hydrolysis upon the growth factor. a) Acid hydrolysis. An active nearly lipid-free preparation of the total proteins of egg yolk was hydrolyzed with 5 N sulfuric acid by heating in an autoclave for 16 hours at 15 lb pressure. The sulfate was precipitated with barium hydroxide and the filtrate concentrated to a small volume. This preparation proved to be inactive. Milder hydrolysis of egg yolk protein by autoclaving with 1 N HCl for one hour similarly destroyed the growth promoting activity of the yolk; *b) Alkaline hydrolysis* of the yolk with 1 N NaOH for one hour also destroyed the activity of the yolk; *c) Enzymatic hydrolysis.* Egg yolk was aseptically added to a sterile trypsin solution. Incubation at 37° for 2 days resulted in a loss of activity.

Other possible sources of the growth factor. A number of other possible sources of the growth factor were tested including beef serum, chicken liver and various commercial liver extracts, milk, casein, dried whey, whole dried yeast and yeast extracts, and spinach. A mixture of vitamins and amino acids[§] was tried in total and in part with various combinations and at different levels. None of these supported growth.

Summary. The studies described indicate that the growth factor in egg yolk required by *Donovania granulomatis* is non-diffusible and was destroyed by hydrolysis with acid, alkali or a crude trypsin preparation. The activity was found in the protein fraction of the yolk, and on separation of the yolk proteins, the major portion of the activity was associated with the vitellin. The factor required for growth does not seem to be any one of a number of well known growth factors which were tested, and furthermore, several other materials considered to be good sources of growth factors when used to replace egg yolk in the medium would not support growth of *D. granulomatis.*

Elizabeth Martin and Agnes C. Redfearn gave technical assistance in this study.

[§] The mixture of growth factors contained: adenine 1 mg, guanine 1 mg, uracil 1 mg, xanthine 1 mg, asparagine 1 mg, nicotinic acid 10 μg, inositol 10 μg, p-aminobenzoic acid 10 μg, riboflavin 40 μg, thiamin hydrochloride 40 μg, calcium pantothenate 40 μg, pyridoxin hydrochloride 40 μg, biotin 0.1 μg, folic acid 0.1, μg, L-histidine monohydrochloride 0.3 g, L-lysine monohydrochloride 0.6 g, DL-tryptophan 0.35 g, DL-phenylalanine 1 g, DL-threonine 0.5 g, DL-methionine 0.5 g, L-leucine 0.5 g, DL-isoleucine 0.6 g, DL-valine 0.5 g and L-arginine monohydrochloride 0.8 g.

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Received September 11, 1953. P.S.E.B.M., 1953, v84.

Adrenal Function Tests in Mice Sensitized to Histamine with *Hemophilus pertussis* Vaccine.[*] (20649)

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Both adrenalectomized mice(1) and mice inoculated with *Hemophilus pertussis* vaccine(2) are more susceptible than normal animals to the lethal effects of histamine, of certain bacterial vaccines, and, of anaphylactic shock. Pertussis-inoculated mice can be protected by cortisone from the fatal effects of subsequent injections of histamine or pertussis vaccine(3). Cortisone likewise restores the resistance of adrenalectomized animals to histamine(4). In view of the similar responses of pertussis-inoculated and adrenalectomized mice to the materials mentioned, it seemed possible that pertussis vaccine might have an injurious effect on adrenal function. The measurement of adrenal function in mice by direct assay of the hormone in body fluids is not feasible. However, indirect indices of adrenal function may be employed. Winters, Schultz, and Krehl(5) measured liver glycogen deposition in fasting rats after ACTH as

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[*] Aided by a grant from the National Institutes of Health.

an index of adrenal function. However, in our experiments with both normal and pertussis-inoculated mice, it was found that this method gave variable results. In one experiment, ACTH would cause a significant rise in liver glycogen; the following day, the same dose of ACTH injected in a similar group of animals would have no observable effect on liver glycogen. Much more consistent results were obtained by a procedure which tested the ability of mice in the fasting state to maintain their liver glycogen at reduced atmospheric pressures. Evans has demonstrated that rats fasted for 24 hours at ½ atmospheric pressure do not lose their liver glycogen store as do normal fasted animals (6). Liberation of adrenal steroids was shown to be essential for this endogenous carbohydrate formation(7).

TABLE I. Effect of Pertussis Vaccine Inoculation and Low Atmospheric Pressure on Liver Glycogen of Mice.

Group	Mice (No.)	Pressure (mm Hg)	Liver glycogen* (mg %)
I Uninoculated mice			
a Uninoculated fed	11	A†	1881±357
" fasted	5	"	106± 29
" "	5	380	1272± 75‡
b " fed	5	A†	2212±521
" fasted	5	"	196± 48
" "	6	278	2442±330‡
II Pertussis-injected animals			
Pertussis-inoc. fed	6	A†	2215±300
" " fasted	5	"	380± 98
Idem	5	380	1256±167‡
"	6	278	Died

* Data expressed as mean with S.E.

† A = Atmospheric.

‡ Indicates statistical significance ($p = .01$) when compared with respective fasted controls at atmospheric pressure.

Materials and methods. Adult male white mice weighing 17 to 23 g were employed. The animals were bred in our laboratory and originated from a strain purchased from Tumblebrook Farms. A group of mice was injected with 0.2, 0.1, and 0.1 ml of *H. pertussis* vaccine[†] on 3 successive days. Six days after the last injection, 4 of the pertussis-inoculated mice and 4 uninoculated animals were challenged intraperitoneally with 50 mg/kg of histamine dihydrochloride. If all pertussis-injected mice died and all control uninoculated mice survived, the remaining pertussis-injected animals were considered to be histamine-sensitive. The LD_{50} of histamine dihydrochloride in pertussis-inoculated mice was found to be 4.7 mg/kg, and, in uninoculated mice, 570 mg/kg. Histamine-sensitive and histamine-resistant mice were placed in separate compartments in a glass desiccator. Air was removed by a vacuum pump and the desired pressure in the chamber was obtained by adjusting an air intake valve. Experiments were run at either 380 or 278 mm Hg. At 380 mm Hg, the ventilation through the chamber was 7.9 liters per minute; at a pressure of 278 mm Hg, 5.1 liters per minute. Experiments were performed at room temperature, which varied from 60° to 70°F. Pressures were reduced gradually over a 30-minute period. No food or water was available to the animals during their 24-hour period under reduced pressure. At the end of this time, atmospheric pressure was restored, the mice were taken from the chamber, and their livers were removed under sodium pentobarbital anesthesia. The organs were then weighed, hydrolysed with 30% KOH and analyzed for glycogen by the method of Seifter *et al.*(8). Controls consisted of mice fasted at atmospheric pressure for 24 hours as well as mice at atmospheric pressure which had been fed.

Results. The data in Table I indicate that at an air pressure of 380 (oxygen tension of 76 mm Hg) both pertussis-inoculated and uninoculated mice in the fasting state maintained liver glycogen levels (1256 ± 167 mg % and 1272 ± 75 mg %) significantly greater than the levels (380 ± 98 mg % and 106 ± 29 mg %) of fasted controls at atmospheric pressures. At 278 mm Hg the liver glycogen of uninoculated fasted animals was 2442 ± 330 mg % as compared with 2212 ± 521 mg % for uninoculated fed animals at atmospheric pressure and 196 ± 48 mg % for uninoculated fasted animals at atmospheric pressure. Although the glycogen values of uninoculated fasted animals kept for 24 hours at 278 mm Hg reached levels comparable with fed animals at atmospheric pressure, 6 of 6 pertussis-inoculated mice did not survive 24 hours at 278 mm Hg, the majority of animals succumbing within a few hours after exposure.

[†] Contained 50 billion organisms/ml. Provided by Sharpe & Dohme, Inc.

In 2 succeeding experiments in which equal numbers of pertussis-inoculated and uninoculated mice were placed in the low pressure chamber at 278 mm Hg for 24 hours similar results were obtained. It was found that 17 of 18 uninoculated mice survived a pressure of 278 mm Hg for 24 hours. Only 3 of 18 pertussis-injected animals remained alive under these conditions. The difference between these groups is a significant one ($p = <.01$).

Discussion. It is apparent from these experiments that under moderate anoxic conditions (380 mm Hg) the adrenals of pertussis-injected, fasted mice function as well as those of uninoculated fasted animals with respect to the maintenance of liver glycogen above the fasting level of control mice. However, under more severe anoxic conditions (278 mm Hg) the survival ability of the pertussis-inoculated mouse, like that of an adrenalectomized animal(1), is low.

Although cortical extract will restore the lowered susceptibility of the adrenalectomized animal to the lethal effects of reduced oxygen tension(1), experiments employing ACTH, cortisone and aqueous adrenal extracts in order to increase the resistance of pertussis-inoculated mice to anoxia have thus far been unsuccessful.

Summary. An increase in liver glycogen occurred in both fasting uninoculated and fasting pertussis-inoculated mice when exposed to ½ atmosphere pressure for 24 hours. Exposure at ⅜ atmosphere for 24 hours killed 17 of 18 pertussis-inoculated mice and only 3 of 18 uninoculated animals.

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Received September 11, 1953. P.S.E.B.M., 1953, v84.

Prevention of Toxic Effects of Injected Thromboplastin by "Thromboplastinase".* (20650)

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Intravenous injection of thromboplastic suspensions in experimental animals has been reported to cause ataxia, convulsions and death(1,2). These effects have been prevented by prior injection of several agents such as rabbit serum, heparin, and congo red(4), soybean trypsin inhibitor(5), monosodium alpha tocopherol phosphate, and inositol phosphatide(6). The morbid or lethal effects of injected thromboplastin have been attributed to thromboembolism(1-3).

Our laboratory recently reported on a new bacterial enzyme, "thromboplastinase" which caused a marked reduction of thromboplastic potency of tissue suspensions *in vitro.* Its preparation, properties, and mode of action have been described(7). In this paper we wish to report the effect of "thromboplastinase" on the toxicity of injected thromboplastic suspensions.

Materials and methods. Active, stable, reproducible rabbit brain or human brain thromboplastic suspensions(8) were used except when indicated. The saline extract of acetone-dried tissue contained finely divided, suspended material. Such suspensions were variously treated before injection into animals:

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* Supported in part by a grant from the National Institutes of Health.

1) Boiled thromboplastic preparations were made by keeping the above suspensions in a 100°C water bath for 30 minutes, which reduced thromboplastic potency from 13 seconds to 40 seconds (one-stage prothrombin time). 2) An enzyme-inactivated thromboplastic preparation (△TP) was made by incubating the original suspensions with “thromboplastinase” at 37°C for various periods. Such treatment progressively rendered the thromboplastic suspensions less potent. 3) Active thromboplastic preparation received no treatment other than refrigeration. These 3 types of suspensions were finally centrifuged at high speed (R.C.F. 20,000 G) for 3 hours, and the sediment resuspended to its original volume with 0.85% NaCl. All suspensions were subjected to the Potter homogenizer in an attempt to reduce and control particle size. There was no $CaCl_2$ in the suspensions used for injection. Potency of the thromboplastic suspensions was tested using a modification(9) of the Quick one-stage method, with normal human oxalated plasma. Concentrations of “100%” (*i.e.* undiluted) thromboplastin clotted such plasma in 12-13 seconds, while clotting times of 40 seconds were obtained with suspensions diluted to 1% with normal saline. Experimental animals employed in this study were male albino rabbits weighing about 1½ kg. The injected materials were pre-warmed to 37°C. Rapid injections of “thromboplastinase” were made in one marginal ear vein. Injections of the thromboplastins were made in the marginal vein of the opposite ear. A positive reaction to injected materials usually occurred within 1-2 minutes after injection. All animals were observed for a maximum of 10 minutes, at the end of which time the reaction of those failing to exhibit ataxia and convulsions was considered negative. Two general procedures were employed whereby “thromboplastinase” could effect the toxicity of injected thromboplastic suspensions: 1) Incubation of thromboplastin with “thromboplastinase” (formation of △TP) prior to injection. 2) Injection of “thromboplastinase” into experimental animals prior to injection of active thromboplastin.

Results. Injection of active thromboplastic suspensions was observed to cause ataxia, convulsions and death as others have previously reported. The approximate least toxic dose of the thromboplastic suspensions was determined prior to each experiment. Table I shows a representative toxicity chart for untreated rabbit brain, human brain and boiled human brain thromboplastin. The least lethal dose in all cases was found to vary between ½ cc/kg body wt and 1 cc/kg body wt, depending on the materials used.

TABLE I. Showing Toxicity of Rabbit Brain, Human Brain and Boiled Human Brain Thromboplastin; note that boiled human brain thromboplastin with very low clotting potency was approximately as toxic as untreated thromboplastin.

Material	Clotting time, sec.	Dose, cc/kg	# animals	Deaths
Rabbit*	12	.25	2	0
		.5	8	0
		.75	4	2
		1.0	11	10
		1.25	4	4
		1.5	2	2
		2.0	4	4
		3.0	4	4
Human*	12	.25	2	0
		.5	6	4
		1.0	4	4
		1.5	2	2
Boiled human*	40	.25	2	0
		.5	2	0
		1.0	2	2

* Brain thromboplastin.

TABLE II. Comparison of the Toxicity of Fresh and Enzyme-Inactivated Thromboplastin at Various Doses.

	Materials injected			
	Fresh rabbit brain thromboplastin CT* 12″		Enzyme inactivated thromboplastin CT 40″	
Dose, cc/kg	No. rabbits	Deaths	No. rabbits	Deaths
½	4	0	—	—
¾	4	2	2	0
1	4	3	2	0
1¼	4	4	—	—
2	4	4	2	0
2½	—	—	2	0
3	4	4	2	0
4	—	—	2	0
5	—	—	2	0

* CT = Clotting time.

In Table II it can be seen that prior incubation of thromboplastin with “thromboplastinase” resulted in a △TP which was nontoxic when subsequently injected. The toxicity

TABLE III. Results of Typical Experiment in Which Thromboplastin, 1 cc/kg Body Weight, Was Injected Intravenously 5 Min. after Administration of the Enzyme.

Inj.	Conc. thromboplastinase, mg/kg	Rabbits	No. of fatalities
Intrav.	5 mg/kg: heat inactivated	3	3
	0.85% NaCl	2	2
	1 mg/kg	2	2
	2	2	2
	3	2	2
	5	5	0
	10	2	0
Intramusc.	5	2	2
Subcut.	5	2	2

of "thromboplastinase" was very low. It was tolerated in doses up to 10 times that required to exert a protective effect. In such doses, its effect on blood clotting (One-stage), blood pressure, and smooth muscle tone of the experimental animal was negligible. When active "thromboplastinase" was administered before the injection of human brain thromboplastin, no deaths occurred after an effective "thromboplastinase" dose was reached. Table III shows a typical experiment where the injection of "thromboplastinase" was followed in 5 minutes by an injection of 1 cc/kg body wt rabbit brain thromboplastin. It can be seen that 5 mg/kg of the crude enzyme was sufficient to prevent toxic effects. Similar results were noted using human brain thromboplastin and boiled human brain thromboplastin as toxic agents.

Intramuscular and subcutaneous injections of "thromboplastinase" exerted no protective action in the 5 mg/kg concentration. Heat-inactivated "thromboplastinase" exerted no protective effect.

The interval between the injection of "thromboplastinase" and the subsequent injection of thromboplastin necessary for effective protection from the lethal effects of thromboplastin varied with the enzyme preparation. In Table IV the effective time of one "thromboplastinase" preparation is seen to be 90 seconds. Other "thromboplastinase" preparations have exhibited the protective effect when only 15 seconds were allowed to elapse between injections of the enzyme and thromboplastin.

Discussion. The observation that injection of thromboplastically active tissue suspensions are toxic to experimental animals has been confirmed on rabbits using tissue from the same species and from other species. No gross difference between species was observed when their thromboplastic potency was approximately equal. It was striking, however, to note that boiled human brain thromboplastic suspension with almost all thromboplastic activity lost still retained its full toxicity as an injected suspension. Chargaff has presented evidence that thromboplastin is a phospholipoprotein(10). One interpretation of the effect of boiling such thromboplastic suspensions is that although the protein moiety was probably denatured, the lipid moiety remained effective. If this were so, one might be led to conclude that the lipid moiety played an essential role in the toxic reactions initiated by injection of thromboplastic suspensions, while the protein portion of the phospholipoprotein was either non-essential or could be replaced by some material *in vivo*. Unless some reservation such as the latter were true, it would appear that there is no necessary correlation between thromboplastic potency and toxicity, since it has been

TABLE IV. Effect of Time Interval between Intravenous Injection of Thromboplastinase (5 mg/kg) and Intravenous Injection of Human Brain Thromboplastin (Clotting Time 13 Seconds) on Toxic Reaction to Thromboplastin.

Interval between "thromboplastinase" and thromboplastin inj., sec.	Result
0	Both developed ataxia, convulsions and death
30	*Idem*
45	1 showed no reaction 1 developed ataxia, convulsions, paralysis, death
60	1 showed no reaction 1 developed ataxia, paralysis, but recovered
90	Both no reaction
120	"
150	"
180	"

Injections of 5 mg/kg crude "thromboplastinase" followed at intervals by least toxic dose of human brain thromboplastin (clotting time 13 sec). 2 rabbits in each exp.

shown that poorly potent thromboplastic suspensions (boiled thromboplastin) can have the same approximate minimal lethal dose as potent thromboplastic suspensions. On the other hand, 2 tissue suspensions of equally poor thromboplastic potency have been shown to have widely divergent least toxic doses: boiled thromboplastin, clotting time = 40″ or more which was highly toxic: △ thromboplastin, clotting time = 40″, which was non-toxic.

The fact that thromboplastic suspensions incubated with "thromboplastinase" lost both their thromboplastic potency as well as their toxicity would seem to support the interpretation that these two properties are functionally correlated. It has been shown elsewhere(11) that "thromboplastinase" probably exerts its action on the lipid moiety of thromboplastin which seems to be associated with the Folch inositol phosphatide fraction. It has been further demonstrated that as a result of "thromboplastinase" action, an organic phosphorus moiety is split from thromboplastin (7).

It is noteworthy that boiling thromboplastin does not lead to a diminution of the amount of phosphorus splittable by "thromboplastinase"(11). This observation would seem to support our interpretation (above) that the lipid moiety of thromboplastin is essential for the production of toxic reactions.

The manner in which pre-injected "thromboplastinase" protects animals against the toxic effects of injected thromboplastin is difficult to explain at present, in view of the rapidity with which the "thromboplastinase" afforded protection. Thromboplastinase *in vivo* seems to exert its protective action almost immediately. *In vitro*, however, destruction of thromboplastin by "thromboplastinase" in the concentrations used followed a much slower course.

Although we cannot interpret this effect, it seems clear that "thromboplastinase" is effective in preventing in rabbits the lethal effect of injected thromboplastin by a mechanism that awaits clarification.

Summary. 1. Loss of potency of tissue thromboplastic suspensions due to "thromboplastinase" action results in a concomitant loss of toxicity. Loss of potency by boiling does not alter the toxicity of thromboplastin. It would appear that the lipid moiety of thromboplastin may be essential for the production of toxic reactions. 2. Prior intravenous injection of "thromboplastinase" into rabbits protected the animals against the otherwise morbid or lethal effects of subsequent injections of a minimal toxic dose of thromboplastin. Heat-inactivated "thromboplastinase" did not induce this protection. 3. Active "thromboplastinase", in doses up to 10 times that needed for protection, was observed to cause no untoward reactions in the animals. 4. The mechanism of the protective effect *in vivo*, seems to be different from the *in vitro* destruction of thromboplastin by "thromboplastinase". The nature of this effect is presently under investigation.

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Received September 14, 1953. P.S.E.B.M., 1953, v84.

Comparison of Hypotensive and Emetic Dosages of Cryptenamine and Other Veratrum Extracts in Humans.* (20651)

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Various alkaloids of the Veratrum group have been shown to exhibit a close relationship between the amount required to lower blood pressure and that required to produce emesis.(1) Recently, however, the alkaloid Cryptenamine, isolated from veratrum viride, was shown in animal investigations to have a ratio of approximately 4:1 in regard to hypotensive and emetic dosages.(2) It was decided, therefore, to determine whether this alkaloid exhibited a similar divergence between the hypotensive and emetic dose in the human.

Materials and methods. Fifty-one hospitalized patients received various extracts of veratrum viride administered intravenously, and 386 patients received the same extracts intramuscularly. In the intravenous group, 21 patients received Cryptenamine and 30 patients received mixtures of the amorphous alkaloids of veratrum viride (15 received Vergitryl, and 15, Veriloid). The diagnoses included toxemia of pregnancy (29), hypertensive encephalopathy (14), and acute glomerulonephritis (8). When given intravenously, 1 mg of each Veratrum extract was diluted with 19 cc of 5% dextrose in water, and the solution was given at a rate of 1 cc or .05 mg per minute until the first 20 mm reduction in systolic or 10 mm reduction in diastolic pressure occurred. Additional Veratrum was not given for a 2 minute period in order to avoid a precipitous fall in blood pressure. If, after waiting 2 minutes, no further reduction in arterial pressure occurred, administration was continued at a rate of .05 mg per minute, stopping at the first subsequent sign of hypotension. In eclamptic patients, administration was continued until the blood pressure fell below 140/90 mm Hg, and in other cases until the pressure fell below 160/110 mm Hg. Dosages were resumed whenever the blood pressure rose above these levels, and treatment was continued for from 1 to 5 days using this intermittent intravenous technic. Of the 386 patients who received these Veratrum extracts intramuscularly, 97 were given Cryptenamine, 233 received Vergitryl, and 56 received Veriloid. All the patients were pregnant; 283 were diagnosed as toxemia of pregnancy, 103 as hypertensive vascular disease. The dosage used was 0.5 mg repeated every hour if necessary to keep the blood pressure under 140/90 mm Hg. The clinical results are given in detail elsewhere (3,4).

Results. The incidence of vomiting following intravenous injection of the various extracts is given in Table I. Following the initial injection of Cryptenamine, 4 of the 21 patients (21%) vomited. Emesis occurred after the initial injection in all of the patients treated with Vergitryl and in all of those treated with Veriloid. Nine patients (43%) of the group given Cryptenamine developed vomiting when repeated injections of the drug were given. Fourteen patients (93%) of the Vergitryl group and 13 patients (87%) of the Veriloid group manifested vomiting on repeated intravenous administration. The average dose of the 3 drugs was essentially the same.

In contrast to these results observed after intravenous injection, the incidence of vomiting observed following intramuscular administration was essentially the same with each of the 3 extracts of veratrum viride (Table II).

Discussion and conclusions. Clinical experience with purified alkaloids of veratrum prior to the synthesis of Cryptenamine showed that there was a relatively low incidence of vomiting following intramuscular administra-

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* This investigation was supported by research grants from the Washington Heart Assn., Squibb Institute for Medical Research, New Brunswick, N. J., Ciba Pharmaceutical Co., Summit, N. J., Irwin Neisler and Co., Decatur, Ill., and Riker Laboratories, Los Angeles, Calif.

TABLE I. Incidence of Vomiting Following Veratrum Extracts Administered Intravenously.

	No. of patients							
Drug	Toxemias of pregnancy	Hypertensive encephalopathy	Nephritis	Avg effective dose, mg	No. patients showing vomiting on 1st inj.	%	No. patients vomiting on repeated inj.	%
Cryptenamine	15	4	2	.3	4	21	9	43
Vergitryl	8	4	3	.3	15	100	14	93
Veriloid	6	6	3	.4	15	100	13	87

TABLE II. Incidence of Vomiting Following Veratrum Extracts Administered Intramuscularly.

Drug	No. patients	Avg dose, mg	No. of patients vomiting	%
Cryptenamine	97	.5	8	7
Vergitryl	233	.5	27	11
Veriloid	56	.5	3	7.5

tion. When a veratrum preparation was given intravenously, however, though a smaller total dose was required to lower blood pressure than when it was given intramuscularly, the incidence of vomiting was greatly increased. It was observed further that vomiting occurred most frequently following the initial intravenous injection.

When given intramuscularly, Cryptenamine resembled the other veratrum alkaloids both in hypotensive effect and incidence of vomiting. When given intravenously, however, it differed from the other alkaloids since moderately rapid administration caused significantly less vomiting particularly following the initial injection. Our data showed that none of the veratrum alkaloids were particularly emetic when their absorption was slowed, but that the receptors initiating emesis were especially sensitive to the sudden higher concentration presented to it when the drugs were given intravenously. It was only in this respect that Cryptenamine differed from the other alkaloids studied. It is concluded, therefore, that the divergence between the hypotensive and emetic dose of Cryptenamine reported in animals also exists in man, and is most apparent after intravenous administration wherein vomiting is common with the other veratrum alkaloids.

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Received September 18, 1953. P.S.E.B.M., 1953, v84.

Effect of Immobilization on Oxygen Uptake of Skeletal Muscle.*† (20652)

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It has been shown(1) that following denervation of the gastrocnemius muscle the cytochrome oxidase activity of this muscle decreased progressively with time. Since these findings did not agree with the results reported by other laboratories(2-4), it seemed desirable to determine the effect of immobilization under conditions which would rule out any contributory effect of fibrillation on the oxygen uptake of the muscle studied.

It also appeared desirable to approach the problem from the opposite angle, *i.e.*, to determine the oxygen uptake of a denervated muscle subjected to constant passive move-

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* Aided by a grant from the National Foundation for Infantile Paralysis.

† Presented at Meeting of American Chemical Society, Los Angeles, Mar. 19, 1953.

TABLE I. Effect of Immobilization on Cytochrome Oxidase Activity of Gastrocnemius and Oxygen Uptake of Diaphragm.

		μl O_2 taken up/mg wet wt of tissue/hr							
		Normal gastrocnemius			Tenotomized gastrocnemius				
Days after operation	No. of rats	Min	Max	Mean	Min	Max	Mean	Mean difference*	P
0	5	9.13	13.47	11.33	9.73	12.87	11.60	+ .2 ± .5	<.80
3	8	11.00	14.13	12.53	8.47	11.33	9.87	—2.7 ± .3	<.001
5	7	8.00	12.27	10.80	6.33	9.33	7.93	—2.9 ± .3	"
10	7	7.27	11.80	9.73	3.60	9.80	5.80	—3.9 ± .2	"
15	7	7.07	12.80	9.80	4.47	7.27	5.60	—4.1 ± .8	<.01
20	9	9.13	14.60	11.47	4.20	9.93	7.40	—4.1 ± .5	<.001
		Q_{O_2} normal diaphragm (0.1% glucose)			Q_{O_2} denervated diaphragm				
0	21	5.60	8.94	7.31	5.17	8.51	7.38	— .07 ± .15	<.70
5	12	7.08	8.94	7.62	6.29	9.00	7.30	— .30 ± .18	<.20
10	11	6.27	8.84	7.53	5.90	8.02	6.46	—1.07 ± .29	<.01
15	11	4.65	11.03	7.03	4.49	8.01	5.69	—1.34 ± .39	"
20	11	6.38	9.56	7.56	4.40	7.11	5.59	—1.97 ± .31	<.001
		Normal diaphragm with 0.2% glucose			Normal diaphragm without glucose				
0	26	5.45	11.70	8.09	5.08	11.36	7.91	+ .25 ± .14	<.10

* Mean of differences between normal and tenotomized gastrocnemius, normal and denervated diaphragm, and normal diaphragm with and without glucose for each animal ± $\frac{\text{stand. dev.}}{\sqrt{n}}$.

ment and compare the changes in oxygen utilization under these conditions with the changes observed following tenotomy with more or less complete immobility.

Experimental. Tenotomies were performed on male mature albino rats as previously described(5). Cytochrome oxidase determinations were carried out by the method described earlier(1). Unilateral denervation of the diaphragm was performed on young rats (60-140 g) by making a ventral incision in the neck, parallel to and approximately over the external jugular vein. The carotid artery and the vagus nerve were exposed and traced caudally to the brachial plexus. The phrenic nerve, arising from the fifth cervical root, runs along the ventral surface of the brachial plexus in close proximity or sometimes even buried in the muscles close to the vertebral column. It may usually be seen with little difficulty as a very thin line coursing caudally across the brachial plexus. The respiration of the animals was examined to make sure that it was symmetrical, the phrenic nerve was grasped with fine forceps and evulsed. The respiration of the rat can immediately be observed to change and it is possible to press one's fingers under the ribs on the 2 sides simultaneously and feel the firm contraction of the diaphragm on the one side and the lack of contraction on the other. At stated time intervals the rats were sacrificed by decapitation and the diaphragm removed as quickly as possible, taking care to avoid traumatization, and immediately placed in an ice-cold buffer solution(6), containing 0.1% glucose. Without removal from the buffer the diaphragm was divided along the midline and trimmed to remove the central tendon and connective tissue. Each hemidiaphragm was then immediately transferred to a chilled Warburg vessel containing the same buffer. The air in the reaction flask was displaced with oxygen. After temperature equilibration of the vessels for 15 minutes, the oxygen uptake was measured at 10-minute intervals for one hour at 37°C. Each hemidiaphragm was then carefully blotted with filter paper and quickly weighed. For dry weight determination each hemidiaphragm was heated in an oven at 100°C to constant weight.

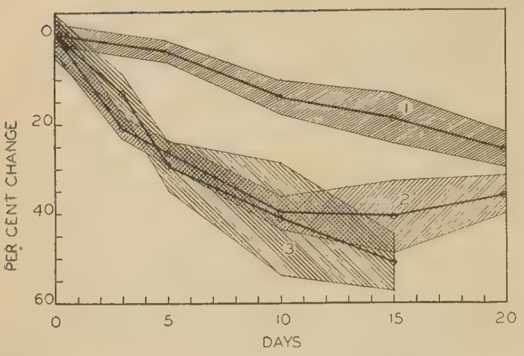

FIG. 1. Effect of immobilization on oxygen utilization on rat muscle. Curve 1: Rate of oxygen uptake of hemidiaphragms following denervation. Present study. Curve 2: Cytochrome oxidase activity of homogenates prepared from gastrocnemius muscle following tenotomy. Present study. Curve 3: Cytochrome oxidase activity of homogenates prepared from gastrocnemius muscle following neurotomy. See reference 1. Shaded areas represent the magnitude of the stand. error of the points of each curve.

Results. In interpreting the results of the present study it should be pointed out that in each case the immobilized muscle was compared with the contralateral normal muscle of the same animal. In the normal animal the activity of the cytochrome oxidase system is relatively constant for comparable muscles as shown in Table I. Immobilization of the gastrocnemius muscle, either by neurotomy or by tenotomy, produces a significant and progressive depression of the cytochrome oxidase system (Fig. 1). The present study, therefore, confirms previous work(6) and shows that changes in the muscle following immobilization follow a similar course irrespective of the manner by which it is produced. These findings, therefore, indicate that fibrillation which accompanies neurotomy is not the major cause of the atrophy which follows sectioning of the motor nerve supply as has been suggested(2). Following the 10th day postoperatively the activity of the cytochrome oxidase system seems to increase gradually in the case of the tenotomized muscle (Fig. 1, curve 2), whereas, the activity of the denervated muscle (curve 3) continues to decrease. This difference may be due to the fact that the severed tendon becomes reattached much sooner than the cut nerve regenerates. Similar observations have been made previously in this laboratory and elsewhere.

Table I also shows that the Qo_2 of the denervated diaphragm decreases progressively and significantly with time after denervation although it is subjected to constant passive movement during the process of breathing. Several investigators(7) have attempted with varying success to influence the rate of atrophy following neurotomy by passive exercise or electrical stimulation, but in the diaphragm of the rat at least, as the present study shows, constant passive exercise does not prevent the eventual decline in oxygen uptake and therefore, presumably will not prevent the ultimate atrophy of the muscle. In Fig. 1, the 2 curves representing the cytochrome oxidase activity of the immobilized gastrocnemius muscle of the rat show the most rapid decline in enzyme activity during the first 5 days following immobilization. The oxygen uptake of the denervated diaphragm (curve 1) on the other hand shows no significant decrease until after the fifth day. Studies are planned to determine if this difference in behavior is due to a possible beneficial result of passive movement or due to the different methods of evaluating oxidative metabolism.

While these studies were in progress Gruber *et al.*(8) reported that the oxygen uptake of the hemidiaphragm, denervated 10-14 days, was about 22% higher than that of the normal control in the absence of glucose, but they found no difference when 0.2% glucose was added to the medium. Table I shows that our results do not support these findings but are in accord with those reported by Gilmore *et al.*(9) and by Paasch and Reinwein(10).

Summary. 1. Following tenotomy of the gastrocnemius muscle of the rat the cytochrome oxidase activity of the muscle decreases progressively. 2. The oxygen uptake of the denervated hemidiaphragm of the rat decreases with time of denervation. This suggests that in this tissue at least passive exercise does not prevent the decline in oxygen uptake, although it may retard it somewhat. 3. The Qo_2 of hemidiaphragms measured in a medium containing 0.2% glucose does not differ significantly from the Qo_2 of hemidiaphragms measured in a glucose free medium.

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Received September 22, 1953. P.S.E.B.M., 1953, v84.

Pharmacologic Differentiation Between Epinephrine- and HGF-Hyperglycemias: Application in Analysis of Cobalt-Hyperglycemia.*† (20653)

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Considerable interest has centered upon the hyperglycemic-glycogenolytic factor (HGF) and its putative role as a hormone. Since HGF is found in association with the alpha-cells of the Islets of Langerhans, definitive tests for a true hormonal role of HGF should be greatly simplified by the recent observations(1,2) that cobaltous chloride selectively damages the alpha-cells. Contrariwise, the similarity in action of HGF and epinephrine (and sympathetic nerve activity) is so pronounced that methods must be at hand to distinguish between these hyperglycemic agents. The hyperglycemia produced by each of these agents is related to an activation of hepatic phosphorylase activity(3). Conditions which interfere with the action of HGF affect equally the action of epinephrine(3,4). Our observation that the adrenergic-blocking drug dihydroergotamine (DHE) prevents the effect of epinephrine on liver slices, but does not interfere with the effect of HGF, has led to a practical differentiation of these two hyperglycemic agents *in vivo*.

This paper presents evidence for the selective blockade of the action of epinephrine on glucose liberation by liver slices and on blood sugar of the intact rabbit, under conditions which allow HGF to produce its usual effects. The information obtained on the intact rabbit has been applied to determine the mechanism of the hyperglycemia which follows the administration of cobaltous chloride, an agent which selectively damages the cells in which HGF is found. The maintenance of normal blood sugar level in rabbits was not influenced by the conjoint effects of damaging the alpha-cells with cobaltous chloride, fasting, and subsequent treatment with DHE.

Methods. Rabbit liver slices were tested as described by Sutherland and Cori(5). The tissues were usually incubated with DHE for 30 minutes before addition of the glycogenolytic agents. In a few cases DHE itself produced a small but significant glycogenolytic effect. For *in vivo* studies rabbits were fasted 20-24 hours before tests. Blood samples were obtained from the marginal ear veins. Glucose determinations were made *ad modum*

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* This work was supported in part by a research grant from the National Heart Institute, of the National Institutes of Health, Public Health Service.

† This paper was presented in part at Fall Meeting of Society for Pharmacology and Experimental Therapeutics, Yale University, New Haven, Sept. 7-9, 1953.

TABLE I. Effects of DHE on the Epinephrine- and HGF-Activation of Glucose Production by Rabbit-Liver-Slices.

1 ml 0.2 ml	Sal. P. Sal. P.	Sal. P. EPI	DHE Sal. P.	Sal. P. Epi	DHE Epi	Sal. P. HGF	DHE HGF
			mg glucose/g/90 min.				
Exp. 1	10	28	15	24	12	21	19
2	9	25	9	22	17	21	21

Rabbit-liver-slices were incubated 90 min., in O_2, at 37°C, in mixture of 4 parts 0.9% NaCl and 1 part 0.1 M sodium phosphate buffer at pH 7.4 (Sal. P.). During first 30 min. the slices were incubated in 1 ml of either Sal. P. or Sal. P. containing 1.2:100000 DHE, and then 0.2 ml of Sal. P., or of l-epinephrine bitartrate (1:100000, EPI; 1:1000000, Epi) or of 1:50000 HGF were added as indicated in table. The higher concentration of epinephrine elicited a maximal response which would also be observed with higher concentrations of HGF. Figures are averages of duplicate experiments.

TABLE II. Effects of DHE on Epinephrine- and HGF-Hyperglycemias.

		Avg blood sugar (mg %) ± S.E.			
		Time before		Time after hyperglycemic agent	
	No. of animals	−30′	0′	30′	60′
Epinephrine	8		101 ± 1	144 ± 7	139 ± 5
DHE + epinephrine	7	101 ± 2	105 ± 3	111 ± 7	113 ± 6
HGF	8		105 ± 3	145 ± 9	136 ± 5
DHE + HGF	10	99 ± 3	100 ± 3	142 ± 11	138 ± 10

Nelson(6). The HGF preparation[‡] which was used was stated to have a potency of 1 unit in 0.5 μg and less than 0.005 unit of insulin activity per mg. This preparation was named 'glucagon' by Staub *et al.*(7). Some preliminary studies were done using an amorphous insulin preparation (22 units per mg).[‡] Injections were made intravenously. The dosages in mg per kg of the drugs injected were as follows: l-epinephrine bitartrate, 0.03; dihydroergotamine tartrate, 0.2; HGF, 1.0; $CoCl_2 \cdot 6H_2O$, 40. When DHE was used, it was injected 30 min. prior to the injection of the hyperglycemic agent.

Results. Differential blockade of HGF and epinephrine in vitro and in vivo. In order to determine whether DHE would interfere with the action of HGF upon liver slices, we have tested concentrations of HGF which produced much less than a maximum effect on glucose production. These conditions avoid the situation in which a large excess of HGF could effectively suppress the action of a reversible blocking agent. In Table I it may be seen that when a concentration of DHE was used such that the glycogenolytic effect of a sub-maximal concentration of epinephrine was partially or completely blockaded, the effects of an equi-active concentration of HGF were not diminished. A preliminary note mentioned these *in vitro* results(4). The first indication we obtained that HGF was not antagonized by DHE was in some student demonstrations on rabbits in which DHE was used to potentiate the hypoglycemic effects of insulin. When these rabbits showed evidence of hypoglycemic shock, epinephrine was of no benefit, whereas an amorphous insulin preparation (heated in alkaline solution to destroy insulin activity, but not HGF activity) appeared to produce a marked improvement in the condition of the insulin-treated animals.

A group of fasted rabbits were tested for their blood sugar response to the purified HGF preparation and to epinephrine, with or without prior treatment with DHE. The results are summarized in Table II. These results clearly demonstrated that the HGF-hyperglycemia was resistant to blockade by a dosage of DHE which completely suppressed epinephrine-hyperglycemia. These results indicated that a clear differentiation of the hyperglycemic effects of HGF and epinephrine could be made with the use of DHE. Since

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[‡] Generously supplied by Eli Lilly Research Laboratories, Indianapolis, through the courtesy of Dr. K. K. Chen.

TABLE III. Effect of DHE on Cobaltous Chloride Hyperglycemia.

	No. of tests	Avg blood sugar (mg %) ± S.E.				
		Time before		Time after cobaltous chloride		
		−30′	0′	30′	60′	120′
$CoCl_2$	7		95 ± 4	144 ± 12	164 ± 16	132 ± 9
DHE + $CoCl_2$	7	95 ± 5	95 ± 6	95 ± 6	98 ± 7	94 ± 8

the HGF-hyperglycemia was not influenced by DHE, the release of HGF from stimulated or damaged alpha-cells should result in a hyperglycemia in the DHE-treated rabbit.

Blockade of cobalt-hyperglycemia by DHE. An interesting application of the above information was suggested by a recent paper on the effects of cobaltous chloride on the blood sugar and alpha-cells of the pancreatic islets by Goldner, Volk, and Lazarus(1). The hyperglycemia produced by cobalt was not limited to the first administration, but occurred on repeated administration at a time when the alpha-cells were severely damaged. If this hyperglycemia were due to the direct or indirect stimulation of the adrenal medulla or to the stimulation of sympathetic nerves to the liver, then pretreatment with DHE should interfere with cobalt-hyperglycemia. On the other hand, a liberation of HGF even upon the first administration of cobalt should produce a hyperglycemia resistant to DHE. The results of administering cobalt with or without pretreatment with DHE on the blood sugar of fasted rabbits are summarized in Table III. These data include cross-over tests in which the rabbits which were pretreated with DHE upon the first administration of cobaltous chloride, were not given DHE before the next administration of cobalt and *vice versa*. It is quite evident from these data that cobalt-hyperglycemia is the result of a sympathetic discharge.

DHE on blood sugar of fasted, cobalt-treated rabbits. Following single or repeated treatment with cobaltous chloride sufficient to destroy the alpha-cells of the Islets of Langerhans(1) rabbits were fasted for 18-20 hours. After blood was obtained for control blood sugar level, DHE was injected. Blood samples were obtained over a period of 3 hours. The blood sugar was at the normal fasting level and did not change during this experimental period in spite of the supposed absence of HGF-producing cells and the blockade of sympathetic nervous activity on the liver.

Discussion. The selective blockade of epinephrine action without interference with the action of HGF implies a different mode of action of the two agents on the liver. Since both glycogenolytic agents produce their final effects by activating phosphorylase, the fact that selective blockade of one of them is possible would make their actions on the same site unlikely. It is evident that neither of the hyperglycemic drugs act directly upon phosphorylase, at least not in homogenized tissues (3). If several mechanisms for the reactivation of phosphorylase are available, it must be that epinephrine acts on one and HGF on another. A likely hypothesis is that epinephrine must first combine with its specific receptor on the cell in order for it to eventually exert its effects on glycogenolysis. This receptor is not used by HGF and thus only epinephrine action is blockaded by DHE.

There are few substances which increase the glucose production of liver slices, although several substances increase glycogenolysis without increasing glucose production. In the intact animal numerous chemicals and procedures increase blood sugar by an action on the liver. Since it is now established that even anoxia does not directly increase glucose production by liver slices(5), it would be well to determine whether agents reported to produce hyperglycemia are influencing blood sugar directly, or simply producing an effect through the mediation of the sympathetic nervous mechanism, or possibly by the liberation of HGF. To a limited extent this attitude has been accepted. Adrenalectomy is not sufficient to rule out an effect through the sympathetic nervous system. With the availability of a simple test such as the one used in this series of experiments, blood sugar effects may be analyzed quite readily.

It would appear that the technic of destruction of the alpha-cells with cobaltous chloride and the blockade of sympathetic effects with DHE (or related substances) should lend itself to a study of the mechanism of blood sugar control and the specific need for these hyperglycemic agents under varied conditions.

Summary. 1. DHE did not interfere with the glycogenolytic effect of HGF on rabbit liver slices or on hyperglycemia in intact rabbits. Comparable effects of epinephrine *in vitro* or *in vivo* were antagonized by DHE. 2. The hyperglycemia induced by cobaltous chloride, which destroys alpha-cells of the pancreatic islets, was prevented by DHE. This hyperglycemia is attributable to sympathetic nerve stimulation. No evidence for the release of HGF was obtained. 3. Blood sugar was maintained at a normal level in rabbits pretreated with cobalt, then fasted and injected with DHE to limit sympathetic action on the liver.

We are indebted to Miss Josephine Stevenson for technical assistance in these studies.

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Received September 22, 1953. P.S.E.B.M., 1953. v84.

Effect of Inositol Feeding on Inositol Phosphatides and Other Lipids of Human Blood Plasma.* (20654)

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Concerned by the role of atherosclerosis in producing hearing loss and labyrinthine dysfunction, it occurred to one of us that a lipotropic factor might be of some clinical value. A study of such possible effects of inositol was initated and has continued for the past several years. Biochemical as well as clinical methods were included. A method has been developed for the determination of lipid inositol in various tissues including blood plasma (1). Results of the determination of the effect of inositol ingestion on the concentration of plasma lipid inositol and several other plasma lipids are summarized in this paper.

Methods. All of the patients reported in this study showed varying degrees of high tone deafness and ranged from 34 to 68 years of age. One initial blood sample was obtained before oral inositol was started. The values for the various lipid components in these samples are in the normal range for the most part and the group could not be considered lipemic. The patients then all received 1 g of inositol† 3 times daily. Blood samples were obtained at varying intervals thereafter and averaged 2 samples per patient. The duration of inositol feeding in the patients reported ranged from 1 to 12 months. None of the patients reported any untoward symptoms or reactions from the inositol. All blood samples were obtained before breakfast between 9 and 9:30 a.m. 50 ml of blood was withdrawn into a centrifuge bottle containing 2 ml of Wintrobe's oxalate. The lipid extracts of the plasma were prepared, purified, and analyzed for total lipid phosphorus and inositol by methods reported previously(1,2). Total fatty acids were determined titrametrically by a modification of the method of Stoddard and Drury(3) on the purified extract.

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* Supported by a Grant from the Life Insurance Medical Research Fund.

† The inositol was generously supplied by the Corn Products Refining Co., Argo, Ill.

TABLE I. Effect of Inositol Feeding on Lipids of Human Blood Plasma in Thirteen Patients.

	Mean		Range		Mean			
	Initial	Final	Initial	Final	change	S.E.	t	P
Total lipid P	295 *	310	230-389	244-376	+ 5.30 ‡	2.12	2.49	.031
Total lipid inositol	7.4*	7.3	5.5-9.4	4.5-9.8	— .8 ‡	2.82	.28	.8
Lipid inositol:phosphorus molar ratio	.025	.024	.022-.029	.019-.029	— 5.44 ‡	2.40	2.27	.04
Total cholesterol	223 †	242	160-309	183-319	+ 9.63 ‡	3.90	2.47	.033
Free cholesterol	61.9†	67.4	42.2-88.8	48.6-91.3	+ 10.0 ‡	3.60	2.78	.017
Ratio free:total cholesterol	.276	.277	.249-.291	.258-.300	+ .50 ‡	1.23	.408	.7
Ratio total cholesterol: lipid P	.757	.785	.624-.913	.689-1.078	+ 4.23 ‡	3.26	1.30	0.2
Neutral fat fatty acids	349 *	161	71-1054	13-374	—188 §	68.2	2.76	.02
Ratio neutral fat F.A.: total cholesterol	1.48	0.67	0.38-3.98	0.07-1.56	— .806§	.259	3.11	.01

* Values given in μm/100 ml plasma. † Values given in mg/100 ml plasma. ‡ Mean % change. § Mean numerical change.

Total and free cholesterol were determined on a separate aliquot of the plasma by the method of Schoenheimer and Sperry(4).

Results. A summary of the results obtained from 13 patients is given in Table I. In this summary the analyses of the last sample only after inositol feeding is chosen for comparison with the initial pre-inositol blood since it represents the maximum time of inositol intake. Cholesterol (free and total), total phospholipids and inositol phospholipids are calculated as the percent change from the initial sample. Neutral fat fatty acids are a more variable component and are therefore calculated in terms of absolute change in micromoles per cent. The statistical method for evaluation of the data is that of Fischer in which Standard Errors of the Difference are obtained using the initial value as zero. The "t" values are thus obtained as the change from the initial sample divided by the Standard Error of the Difference.

Discussion. Although the series is small it seems established that no real change occurred in the concentration of plasma inositol phosphatides incident to feeding amounts of inositol which are many times greater than the ordinary dietary intake. The ratio of inositol phosphatides to the total plasma phosphatides appears to be quite constant and all the values fall within the range of .019 to .029 in the samples reported. In another group of 6 patients who did not receive inositol and are not reported, the values also conformed to this range.

There appeared to be small but measurable mean increases in total cholesterol, free cholesterol, and total phospholipids. However, these changes are of doubtful statistical significance. These latter lipid components have been measured in several inositol feeding experiments on man and animals by others who reported (a) decreases in total cholesterol (5-8) and phospholipids(7) or (b) little or no change in cholesterol(9-12), phospholipids (10,12) and low density lipoproteins(12). In cholesterol fed rabbits, inositol was reported to be both ineffective(13) and effective(14) in preventing the hypercholesteremia. In old hens inositol reduced cholesteremia(15) but failed in combination with choline to reduce the cholesteremia and general lipemia in chicks fed cholesterol(16) or treated with estrogen (17). It seems clear that no simple relationship, if any, exists between exogenous inositol and blood plasma total cholesterol, free cholesterol or total phospholipids.

In this study, the neutral fat fatty acids decreased with inositol feeding, and more strikingly in the more lipemic patients—*i.e.*, those with initial cholesterol values over 200 mg%. The decreases as recorded appear to be significant. However, caution must be exercised in the interpretation of changes in as highly variable a fraction as the neutral fat. The mean decrease in the "lipemic" group is less than the magnitude of what must be considered a "normal" range for neutral fat. Moreover, no consistant decrease in the neutral fat fraction was observed in cholesterol fed chicks(15) or estrogen treated chicks(16) given inositol with choline. Nevertheless, these data are of considerable interest since at least a part of this fraction is associated with low density lipoproteins(18) which in turn are thought by some to be associated with the development of atherosclerosis. Studies are in progress to further examine such a relationship between inositol and neutral fat.

Summary. A group of 13 patients with high tone deafness was treated with 3 g oral inositol daily for periods of 1 to 12 months. No change resulted in the fasting plasma inositol phosphatide concentration; there were minor increases in total and free cholesterol and total phospholipids, and there was an apparent decrease in neutral fat.

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Received September 28, 1953, P.S.E.B.M., 1953, v84.

Vitamin E Activity of Alpha-Tocopherylhydroquinone and Muscular Dystrophy. (20655)

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It was shown for the first time some 14 years ago that the muscular dystrophy produced in rabbits fed certain natural food diets, purified fat free diets, or cod liver oil, can be prevented or cured by highly purified concentrates of wheat germ oil or by α-tocopherol(1-3). The effect of vit. E on the intense creatinurea exhibited by dystrophic rabbits was dramatic, a precipitous fall in creatine excretion occurring in the first 24 or 48 hours after the initiation of therapy(1). Subsequently it was found that the duration of the fall in creatine excretion caused by single doses of α-tocopherol provided an excellent bioassay for antidystrophic activity, the number of days of suppressed creatinurea being proportional to the size of the dose administered(4). Utilizing this antidystrophic assay in rabbits, it was discovered recently that α-tocopherylhydroquinone is a potent antidystrophic substance(5,6). Indeed the antidystrophic activity of a 5 mg dose given intravenously approximates the activity of α-tocopherol itself. However, the antidystrophic activity of the hydroquinone is somewhat unique, for unlike α-tocopherol it is more potent when administered intravenously than when given orally. Moreover, unlike α-tocopherol, it is not stored, for increas-

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[*] This work has been made possible by a grant from the Association for the Aid of Crippled Children.

ing the dose of α-tocopherylhydroquinone beyond a 5 mg level does not appreciably extend the duration of the response.[†]

These findings suggested to us(6) that α-tocopherylhydroquinone possesses antidystrophic properties *per se*, rather than as a result of its conversion to α-tocopherol in the body. In an attempt to shed further light on this hypothesis, we have investigated the antisterility activity of α-tocopherylhydroquinone in vit. E deficient female rats. Since the hydroquinone is not stored, it was evident that the administration of a single dose at the beginning of pregnancy would not be satisfactory and that repeated injections should be given throughout pregnancy(6). However, the rapidity with which the free hydroquinone undergoes auto-oxidation presented difficulties for such a procedure. Consequently the more stable α-tocopherylhydroquinone disuccinate, which has also been shown to possess antidystrophic activity(7), was employed. This derivative was repeatedly injected via the tail vein throughout pregnancy. The antisterility activity of α-tocopherol administered intravenously was likewise examined. The effect of injected α-tocopherylhydroquinone and its disuccinate on the level of serum tocopherol in dystrophic rabbits was also determined. These experiments and their implications with respect to the *in vivo* activity of α-tocopherylhydroquinone comprise the subject of this paper.

Methods. Female rats of the Sprague-Dawley strain were placed on the vit. E deficient diet of Mason and Harris(8) at the time of weaning. Each rat received 2 drops of percomorph oil orally 3 times a week, and 0.5 mg of a vit. K preparation (Synkamin) intraperitoneally once a week. After the young rats had been maintained on this regimen for 4 months they were subjected to an initial resorption gestation. Following a period of recovery, the animals were placed with a male at Stage I of estrus, and test compounds were injected on the following day when sperm were found in the vaginal smear. Prior to injection, each rat was placed before an infrared lamp for 15 minutes. The animal was then immobilized by wrapping it snugly in a double length of cheesecloth, and its tail was immersed in a water bath at 40-45°C for 3 minutes, following which the test compound was injected into the dilated tail vein through a No. 25 gauge needle. Unless these precautions to insure complete dilatation of the tail vein were taken, cellulitis and necrosis of the tail resulted, rendering the animal unsuitable for further supplementation.

The sodium disuccinate of α-tocopherylhydroquinone,[‡] prepared according to the procedure described earlier(7), was dissolved in either 0.15 M phosphate buffer or in isotonic saline and adjusted to pH 7.4. The volume of a single injection of these disuccinate solutions did not exceed 1.5 ml. Alpha-tocopherol was administered as a suspension in one part of ethanol and 9 parts of propylene glycol. Although the propylene glycol present in as little as 0.05 ml of this mixture produced hematuria within 30 minutes of the intravenous injection, this reaction did not interfere with pregnancy. In all tests the appearance of the placental sign between the 10th and 15th day following insemination and the typical gain in weight were both required as evidence of pregnancy.

The antidystrophic activity of the disodium disuccinate of α-tocopherylhydroquinone was checked by intravenous administration to vit. E deficient dystrophic rabbits. The succinate was again found to have from one-quarter to one-half of the antidystrophic activity, on a molar basis, of the free hydroquinone or of α-tocopherol(7). Serum tocopherol levels were determined by the method of Quaife, Scrimshaw, and Lowry(9).

Results and discussion. In the initial experiments female rats with no detectable toco-

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[†] Frequent administration of small doses of α-tocopherylhydroquinone completely presents or cures dystrophy in the rabbit(5,6). These findings on the rabbit have been extended by Dr. Karl Mason to the prevention and cure of dystrophic lesions in the suckling rat and in the adult hamster by α-tocopherylhydroquinone (Personal communication).

[‡] We are indebted to Dr. Milton Farber and Dr. Ade T. Milhorat for generous supplies of α-tocopherylhydroqinone disodium disuccinate and α-tocopherylhydroquinone.

TABLE I. Antisterility Activity of α-Tocopherylhydroquinone Disodium Disuccinate and α-Tocopherol Injected Intravenously in Vit. E-Deficient Female Rats during Pregnancy.

No. of rats	Supplement	mg per dose	No. of doses	Days between doses*	Total mg inj.	No. of rats delivering
5	α-T.hydroquinone disucc.†	8	6	2	48	0
4	" "	8	10	1	80	0
8	" "	4	16	0	64	0
8	" "	8	16	0	128	0
6	d,l α-Tocopherol‡	3	1		3	6
9	α-T.hydroquinone disucc.	8	19	0	152	9
	+ d,l α-Tocopherol	3	1		3	

* First dose of test compound was inj. on day that sperm appeared in vaginal smear.

† α-tocopherylhydroquinone disodium disuccinate. 8.04 mg of this material are equivalent on a molar basis to 5.0 mg of α-tocopherol.

‡ Kindly supplied by Merck and Co., Rahway, N. J.

pherol in their serum were injected by tail vein with 8 mg of α-tocopherylhydroquinone disodium disuccinate on every third day or on alternate days throughout pregnancy. In neither case was this treatment effective and typical resorption pregnancies resulted (Table I). In the next experiment 8 similar rats were injected daily with 4 mg of the disuccinate. By the end of the 16th day it was evident from the sharp decline in the weight curves that resorption of the young had occurred in each of these animals. After a period of rest these same 8 rats were mated again and injected with 8 mg of the disuccinate *daily* for 16 days, or with a total of 128 mg, equivalent, on a molar basis, to 80 mg of α-tocopherol. None of these animals gave birth to young (Table I) and all showed the loss of weight, beginning at about the 16th day, that is typical of resorption.

In contrast to the results obtained with the hydroquinone the intravenous administration of a single 3 mg dose of α-tocopherol on the first day of pregnancy resulted in the birth of living young to each of the rats tested (Table I). Moreover, when the rats who had failed to respond to the hydroquinone were injected with a single dose of α-tocopherol at the beginning of a subsequent pregnancy, living young were born in every instance. Finally, vit. E-deficient rats were given a 3 mg dose of α-tocopherol by mouth on the day of insemination and were then injected daily with 8 mg of the disuccinate for the next 19 days (Table I). All of these rats gave birth to living young, thus eliminating the possibility that trauma caused by repeated injections was responsible for the negative findings obtained with the hydroquinone. The results of these experiments show that α-tocopherylhydroquinone disuccinate is essentially devoid of antisterility activity when administered under the same conditions that led to the discovery of its antidystrophic activity.

It may be calculated on the basis of the foregoing experiments that less than one-tenth of the α-tocopherylhydroquinone disuccinate could have been converted to α-tocopherol in the animal body. Mason(10) has shown that the administration of divided doses of vit. E during the first 10 days of pregnancy is a very satisfactory procedure, and that 0.7 mg of α-tocopherol so administered prevents sterility in a large number of rats. Our rats which were injected with 8 mg of the disuccinate daily received during the first 10 days a quantity of α-tocopherylhydroquinone equivalent on a molar basis to 50 mg of α-tocopherol, yet not one of them gave birth to young. In terms of antidystrophic activity, this amount of the disuccinate is equal to 12-25 mg of α-tocopherol. Consequently it appears that the antidystrophic activity of α-tocopherylhydroquinone cannot be accounted for by its conversion to α-tocopherol in the body.

Additional support for this conclusion was obtained in a study of the level of serum tocopherol produced by the intravenous administration of α-tocopherol, α-tocopherylhydroquinone, and the disuccinate of the latter compound to rabbits with no detectable

TABLE II. Serum Tocopherol 24 Hours after Intravenous Injection of Single Doses of Antidystrophic Compounds in Rabbits with Stage I Dystrophy and No Detectable Serum Tocopherol.

Compound	mg inj.	Serum tocopherol, mg %
d,l α-Tocopherol	5	.5
”	10	1.2
”	10	1.1
”	10	1.0
”	10	1.1
α-Tocopherylhydroquinone	10	0
”	10	0
”	100	0
α-T.-hydroquinone disucc.*	20	0
” ”	20	0
” ”	40	0
” ”	100	0

* α-tocopherylhydroquinone disodium disuccinate.

serum tocopherol. As shown in Table II, when such animals were injected with a single dose of 5 or 10 mg of α-tocopherol, substantial levels of tocopherol were found in the serum 24 hours later. In contrast to these results, the injection of even 10 times as much hydroquinone or hydroquinone disuccinate gave rise to no detectable serum tocopherol. Nevertheless, the latter compounds produced a drop in creatine excretion and marked improvement in the physical symptoms of dystrophy.

Both the results of these latter experiments and of the antisterility assay experiments indicate that α-tocopherylhydroquinone is not converted to α-tocopherol to a significant extent in the body. It appears therefore that α-tocopherylhydroquinone possesses antidystrophic activity *per se,* and is, in effect, an antidystrophic vitamin. The separation of antidystrophic from antisterility activity is a matter of considerable interest for the most ubiquitous response of experimental and domestic animals to vit. E-deficiency is muscle degeneration. This is true even in the rat, our classic and overemphasized example of sterility, for at both extremes of its life span the rat develops severe muscle lesions in the face of vit. E deprivation, and in the interim it is afflicted with muscle weakness and scattered muscle lesions(11). Many other vit. E-deficient animals develop dystrophy in the absence of signs of sterility. For example, rabbits subjected to repeated attacks of muscular dystrophy and finally allowed to succumb to the disease, do not develop testicular degeneration(12,13) and female rabbits with extensive muscle lesions give birth to living but dystrophic young(14).

The dystrophy caused by vit. E deficiency may be prevented or cured by α-tocopherylhydroquinone, which is devoid of antisterility activity; or, by α-tocopherol, which exhibits both antisterility and antidystrophic activity. With respect to the antidystrophic effect of α-tocopherol, 2 possibilities exist; first, that it possesses antidystrophic activity as such, or second, that its antidystrophic activity is due to its conversion in the body to the hydroquinone and that, with respect to this function, it is a provitamin or storage form for the active antidystrophic factor, α-tocopherylhydroquinone. If the second alternative is indeed the case, muscular dystrophy could be caused by any one of several conditions: a deficiency of vit. E, an inability to convert tocopherol to the active hydroquinone, or excessive destruction *in vivo* of this unstable compound. In view of these possibilities and the finding that α-tocopherylhydroquinone is an antidystrophic substance in its own right, the need for a critical and sober examination of its effect on human muscular dystrophies is clearly indicated.

Summary and conclusions. The disuccinate of α-tocopherylhydroquinone has been tested for antisterility activity by daily intravenous injections in pregnant vit. E-deficient rats. This antidystrophic compound was found to be devoid of the antisterility activity associated with α-tocopherol. Moreover, the injection of dystrophic rabbits with large curative doses of α-tocopherylhydroquinone, or its disuccinate, did not lead to the appearance of tocopherol in the serum. Both of these observations indicate that α-tocopherylhydroquinone is not converted to α-tocopherol to a significant extent in the body. It appears therefore that α-tocopherylhydroquinone is an antidystrophic factor or vitamin *per se.* Alpha-tocopherol, on the other hand, is the antisterility vitamin. It may possess antidystrophic activity as such, or alternatively, it may only be the provitamin for α-tocopherylhydroquinone. The separation of antidystrophic from antisterility activity is discussed with relation to the function of vit. E in muscular dystrophy.

The authors wish to express their appreciation for the excellent technical assistance of Mrs. Helen M. Casteel.

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Received October 8, 1953. P.S.E.B.M., 1953, v84.

The Precipitability of Two Modified Proteins.* (20656)

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Precipitin-type reactions, exhibiting specificity between iodinated proteins and arsonobenzeneazoproteins, have been reported for extracts of *Saccharomyces cerevisiae* repeatedly cultured in the presence of such modified proteins(1). From extended attempts to extrapolate this work, it has been learned that such precipitates may be obtained from complex combinations of experimental conditions, in the absence of the yeast. The specificity which was originally observed is believed, therefore, to be non-biological in all or most of the cases studied. That a major factor contributing to the observed precipitin-like reactions and their variation may have been pH was suggested by the fact that precipitin-like behavior was frequent during some periods of experimentation, yet infrequent in others. After many such periods, the former type appeared to be correlative with higher acid content in the samples of ether employed in cytolysis. The finding that some cultures yielded more reactive extracts was probably due to fortuitous acid content rather than to a parallelism between behavior of yeast and the long-known erratic antibody-producing power of animals(2).

Haurowitz(3) has shown that reported instances of precipitin reactions from *in vitro* incubation of serum globulin with acid azoovalbumin(4) is ascribable predominantly to charge effects. In the case of the yeast cultures, however, the more rigorous test of specificity of reaction appeared to be met. The results reported in this paper suggest that such results may appear when the interplay of conditions leads to overlapping, yet in part discrete, pH regions of precipitability for two or more antigens. In the course of these studies, attention was particularly focussed upon the pH factor by the related finding that evaluation of specificity of protease-substrate interaction was dependent upon pH to a degree much greater than that earlier recognized (5-9). In both types of situation, other factors than pH contribute to the effects observed.

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* Journal Paper No. J-2384 of the Iowa Agric. Exper. Station, Project No. 1111, supported in part by the Rockefeller Foundation.

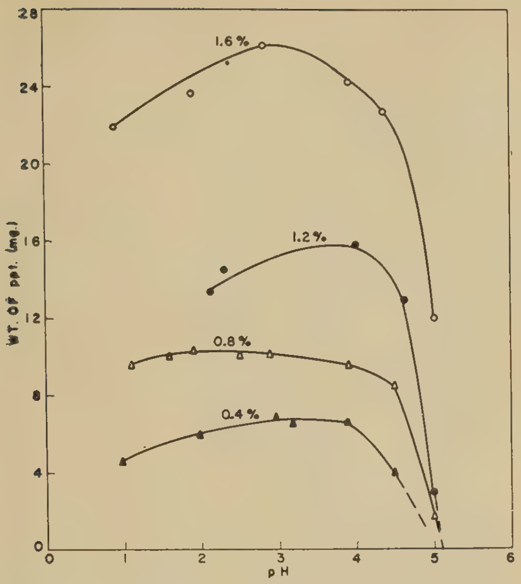

FIG. 1. Precipitation range for iodocasein solutions of different concentrations.

Materials and methods. The iodocasein was prepared by adding a solution of 2.0 g of powdered iodine in 50 ml of 10% potassium iodide solution to a solution of 25 g of Smaco casein in 1 l. of 1 N sodium carbonate solution, allowing the mixture to stand overnight, neutralizing with hydrochloric acid to maximum precipitation, and washing with water. Found: 1.04% I, calculated to a dry weight basis. For testing, the iodocasein was dissolved in 1 N sodium hydroxide solution and the liquid was neutralized with 1 N hydrochloric acid. The arsonobenzeneazocasein was prepared by the method of Boyd and Mover (10) in sodium carbonate solution. Found: 2.0% As, calculated to a dry weight basis. Solutions were prepared in the same manner as for the iodocaseins.

For the *results* of Fig. 1 and 2, concentrations of the iodocasein and arsonoprotein solutions were varied as indicated. In each test, 1 ml of solution of modified protein was diluted with 5 ml of 5% sodium chloride solution and the pH was adjusted with concentrated sulfuric acid added from a micropipet. Precipitates were washed with water, allowed to dry in air, and weighed.

In Fig. 3 the effect of hydrochloric acid may be observed, and is seen to result in curves different from those obtained with sulfuric acid. Although variation in shape of the curve has been observed with each acid, each is characteristic in its behavior.

Discussion. Aside from the effects of protein concentration, pH, and acid type; salt concentration, salt type, and other proteins in the system(3) may be expected to influence the response. Any added protein, such as yeast globulin, might therefore modify the results observed. Indications that conditions of storage and of incubation affect such precipitations have also been noticed.

The exact conditions of Fig. 3 would suggest more frequent precipitation of iodoprotein than of arsonoprotein, whereas the opposite has been recorded(11). The results presented, however, indicate that the phenomena described permit essentially the simulation of

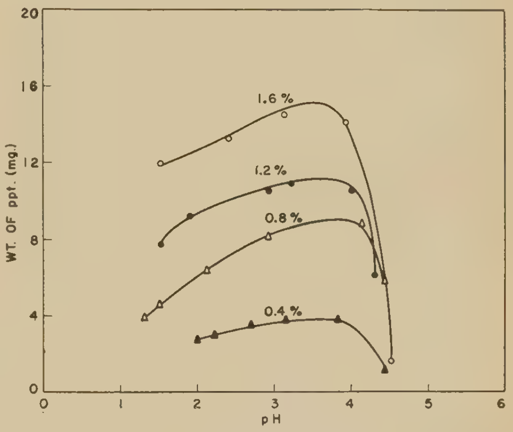

FIG. 2. Precipitation range for arsonobenzeneazocasein solutions of different concentrations.

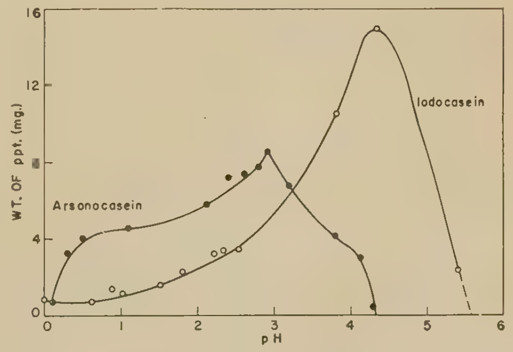

FIG. 3. Relatively discrete pH ranges of precipitation for iodocasein (1.0%) and arsonobenzeneazocasein (1.2%).

specific precipitation, in the absence of cellular extracts.

The effects recorded with artificial antigens, as described here, indicate the need for control of many conditions before either precipitins or specific precipitins can be related to biological causes. Furthermore, the well-known variability in immune response, even from the same organism at different times(12), may stem in part from the operation of such factors. With increasing knowledge of protein precipitation(13), it may be expected that the variability which has so often been noted in the immunochemical literature (*e.g.*, 2,12) may also be greatly diminished.

Marrack, J. R., and Grant, R. A., (*Brit. J. Exptl. Path.*, 1953, v34, 263) record and discuss effects of salt concentration and pH on antigen-antibody reaction.

Summary. Conditions have been studied for the simulation of specific precipitins. The factors which have been shown to operate may explain earlier reports of antibody production from yeast. It is suggested that some of the variability observed in conventional immunological test may have, at least in part, a similar basis in the effect of conditions of the tests upon the precipitability of the proteinaceous antigens.

Thanks are expressed to Mr. Joseph E. Johnson for his assistance.

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Received October 9, 1953. P.S.E.B.M., 1953, v84.

Nutritional Requirements of Clostridium Tetanomorphum. (20657)

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This study was undertaken to determine the nutritional requirements and general metabolic activities of an anaerobic bacterium listed in the Stanford Bacteriology Department collection as strain E-42 of *Clostridium cochlearium*. It was received from Dr. I. C. Hall in 1930 and at that time was designated by him as *Clostridium putrificum*, strain No. 3379. This strain was later identified as *C. cochlearium* by Hartsell and Rettger(1). During the course of our studies on the metabolism of E-42 it became evident that this species is capable of fermenting glucose (or maltose) and produces butyric, acetic, and lactic acids, carbon dioxide, and hydrogen from this sugar. In addition we observed that this strain ferments pyruvic and glutamic acids with the production of the products listed above, together with ammonia from glutamic acid, in approximately the same amounts as reported by Woods and Clifton(2) for *Clostridium tetanomorphum*. Threonine and serine were the only other amino acids fermented at an appreciable rate as evidenced by gas production. These observations indicate that strain E-42 as it is now constituted is a strain of *C. tetanomorphum* rather than of the closely related species, *C. cochlearium.*

Materials and methods. In the growth studies a salt solution consisting of 11.5 g of $Na_2HPO_4 \cdot 12\ H_2O$, 1.0 of KH_2PO_4 and 0.1 of $MgSO_4 \cdot 7\ H_2O$ per l of distilled water plus 2 ml of a trace element solution (Snell, *et al.*, 3) served to supply the inorganic requirements of E-42. Growth occurred in this solution at pH 7.4-7.6 when growth factor and amino acid requirements were supplied by 1% yeast extract and casein hydrolysate respectively, and reducing conditions were developed with 0.1% sodium thioglycollate. The addition of glucose or maltose supported heavier growth, and 0.5% glucose was employed in the test media. In the preliminary studies various growth factors were added in appropriate amounts to the medium described above from which yeast extract was omitted and to which 10 mg each of L-trptophan and L-cystine were added per l to make up for any possible deficiency of these amino acids. The various mixtures were autoclaved and 9.5 ml amounts were distributed aseptically into sterile test tubes. The fluid in each tube was covered with a 2 to 3 mm layer of sterile mineral oil. Before inoculation the tubes were heated in a boiling water bath for 15 min. to expel any dissolved oxygen and then rapidly cooled. Five-tenths ml of an inoculum comprising well-washed cells from 17 to 20 hr cultures was introduced into each tube and the development of turbidity on incubation at 37°C was observed. The amino acid requirements were determined in the same manner, one amino acid in turn being omitted from the basic medium to which no casein hydrolysate had been added. Finally growth experiments were conducted in the basal salt-glucose-thioglycollate medium to which only known amino acids and growth factors were added.

Results and discussion. Little or no growth occurred when either nicotinic acid (or its amide) or pantothenic acid was omitted from the medium and the crop yield was greatly decreased in the absence of biotin. This latter behavior was studied further and it was found that the sample of casein hydrolysate employed contained a trace of biotin. No growth occurred in the absence of biotin when a biotin-free casein hydrolysate was employed as the amino acid source. Choline, *p*-aminobenzoic acid, and thiamine appeared to have a slight stimulatory action on growth but had little or no influence on crop yield.

Little or no growth occurred when either L-glutamic acid or L-tryptophan was omitted from the basal medium devoid of casein hydrolysate. Tyrosine, leucine, and phenylalanine appeared to promote growth to a slight extent but were not essential for growth of E-42.

In further studies it was observed that fair growth of *C. tetanomorphum* occurred in the basal salt-thioglycollate solution containing 0.5% of glucose, maltose, or sodium pyruvate and, in amounts per 100 ml, 75 mg L-glutamic acid, 25 mg L-tryptophan, 20 µg each of nicotinic acid and calcium pantothenate, and 1 µg of biotin.

Summary. Strain E-42, presently identified as a strain of *C. tetanomorphum,* is exacting in its nutritional requirements. Growth will occur in a basal salt-glucose-thioglycollate medium only following the addition of pantothenic and nicotinic acids, biotin, L-glutamic acid, and L-tryptophan. *p*-Aminobenzoic acid, choline, thiamine, leucine, tyrosine, and phenylalanine may exert slight stimulatory action on growth but are not essential for growth of this organism.

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Received October 13, 1953. P.S.E.B.M., 1953, v84.

Use of a Deficient Ration for Chicks in Bioassay of Gonadotrophic Hormones.* (20658)

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Commercial chick rations are frequently used during the study of gonad response to hormone injections(1). Since the chicks gain weight on this ration, the hormone dosage must be sufficient to elicit a testes weight response over and above the normal increase. Some investigators maintain their chicks without food or water over the experimental period, depending on the unabsorbed yolk to sustain life for 96 hours, after which inanition begins (2). This inhibition of growth results in a significant weight increase at lower levels of hormone dosage. However, the term of the experiment must be short and injections made at frequent intervals. Therefore the full potency of each injection is limited and the overall sensitivity may be reduced.

It was observed that a corn-cob meal ration inhibited growth and caused the endocrine glands to remain infantile in size. These conditions allowed for a maximal dosage response and a longer interval between injections. Based upon these considerations a method for testing pituitary gonadotrophic hormones was devised.

Materials and methods. One-day-old White Leghorn cockerels were kept in an electric brooder and maintained either on commercial chick starter ration or on the corn-cob meal ration. The tested pituitaries, with a mean fresh weight of 11.3 mg (range 10.6-11.8 mg), were obtained from male Rockland rats, 10-12 months of age. They were homogenated and diluted to a volume of 1.25 cc in distilled water. Each volume contained one rat pituitary or fraction thereof. The experimental chicks received 5 daily injections into the axillary region with 0.25 cc of the homogenate. The birds were sacrificed on the sixth day and the testes removed and weighed.

Results. The results are presented in Table I. With the chicks maintained on corn-cob meal ration it was possible to show a graded response of chick testes weight to fractions of whole rat pituitary. There was a significant difference between the 1, ¾ and ½ fractions compared to the response of the non-injected chicks. There was no significant difference in the ¼ group when it is compared with the ½ and 0 groups. Therefore the minimum dosage required to elicit a response in chick testes under these conditions was ½ rat pituitary homogenate per chick.

With chicks maintained on a commercial ration the data show that to elicit a significant gonadotropic response a dosage of at least 1 rat pituitary per chick must be administered.

There was no significant difference between

TABLE I. Graded Response of the Chick Testes to Whole or Fractionated Rat Pituitary Injections.

Group	No. of chicks	Ration	Mean testes wt (mg) ± stand. error	Range (mg)
1 pit/chick	8	Corn-cob*	18.2 ± 1.10	15.6–24.4
¾ ”	12	”	14.3 ± .98	8.8–20.6
½ ”	10	”	11.5 ± .65	8.6–14.1
¼ ”	9	”	10.6 ± .93	7.6–14.0
Saline inj.	10	”	9.4 ± .4	8.2–12.0
”	9	Commercial†	18.4 ± 1.0	12.0–21.8
1 pit/chick	9	”	25.6 ± 1.9	16.4–36.0

* Finely ground yellow corn-on-cob meal.

† Chick Starter Ration, Security Mills, Knoxville, Tenn.

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* Published with the permission of the Director of the University of Tenn. Exp. Station.

injections of one or three pituitaries per chick maintained on corn-cob meal ration suggesting that a threshold response to gonadotropic hormones had been reached with injections of one rat pituitary per chick.

Summary. Day-old cockerels were kept on a corn-cob meal ration while testing homogenates of rat pituitaries for gonadotropic hormones. The chicks failure to grow made the testes weight a more sensitive indicator of gonadotropin when contrasted with the cockerels on a balanced ration. With the corn-cob meal ration the chick assay method was significant with ½ of a rat's pituitary.

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Received October 13, 1953. P.S.E.B.M., 1953, v84.

Formation, Flow, and Reabsorption of Cerebrospinal Fluid in Man.* (20659)

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On the basis of data obtained from multiple simultaneous isotopic tracer studies in man, employing various suitable combinations of Na-24, K-42, Cl-38, Br-86, P-32, HTO, D_2O and radio-iodinated human serum albumin, we have been led to a unified theory of cerebrospinal fluid (CSF) formation, flow and reabsorption which is at variance with the classical and currently held concepts. The textbook teaching(1) is that CSF is formed by the choroid plexuses (Dandy, 2) of the cerebral ventricles whence it flows into the spinal and epicerebral subarachnoid spaces. Here it is considered free to circulate until it is finally reabsorbed, presumably largely or wholly at the arachnoid villi (Weed(3)).

We wish to present evidence that CSF is elaborated throughout the cerebrospinal fluid system independently of flow from one area to another. It is derived both from the choroid plexus in the ventricles, and in the subarachnoid space from the vascular bed of the pia. The reabsorption of CSF, in so far as water and electrolytes are concerned, has similarly been demonstrated by us to occur throughout the system independently of flow. These elements of the fluid enter and leave the ventricles and subarachnoid space by direct molecular capillary exchange, and hence may be considered to be in a state of dynamic equilibrium with plasma, similar to that of other fluid compartments of the body.†

Methods of study. After a large number of tracer studies had been done in patients with normal CSF systems, it became evident that a precise compartmental analysis of the data involved equations of a fair degree of complexity, and more variables than could be adequately measured, the most troublesome in this respect being the rate of flow between compartments. Accordingly we set out to limit the variables insofar as possible and to test the validity of a number of possible compartmental models of CSF physiology in a series of critical experiments. To this end, we sought patients for study in whom it would be possible to isolate the main ventricular compartments from the subarachnoid space. Two such patients were found; and the studies presented below, on which our unified concept of the CSF system rests, were carried out in them. These patients had developed complete blockage of CSF outflow from the large lateral ventricles as the result of tumor encroachment on the third ventricle over five years ago. At that time they were restored to a normal state of CSF pressure and dynam-

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* This study was aided by grants from the National Institutes of Health, U. S. Public Health Service, by the Atomic Energy Commission and by the Associated Universities, Inc.

† In this paper, the mechanism of exchange is not considered, i.e., whether by simple diffusion or some form of active transport.

ics by means of a small plastic tube so placed surgically as to bypass the block and restore the flow of CSF from the lateral ventricles to the basal cistern of the subarachnoid space, (the so-called Torkildsen operation). In these last 5 years since operation, their intracranial pressures have remained essentially normal. The neurological signs in one of them (S.L.) had deteriorated somewhat, but the other has remained unchanged, suggesting in both cases a very slowly evolving type of lesion. In patient S.P. at the time of one of our studies, the CSF protein was within the normal range, being 13 mg% in the ventricle and 18 mg% in the lumbar subarachnoid space. The relatively normal status and CSF findings in these patients would seem to be reasonable evidence that the tumor tissue itself is making no significant contribution to the exchanges of water, electrolytes and protein. That any disturbance in the normal mechanism of exchange which may have attended their operations or prior period of hydrocephalus has had ample time for recovery in the intervening 5 years of unimpeded CSF outflow from the ventricles, is evident in the similarity between isotopic appearance curves in the ventricular CSF of these patients and those of more normal patients after intravascular injection(4). Hence, we feel warranted in the tentative conclusion that our studies in these patients provide an approximately quantitative as well as qualitative notion of the CSF mechanisms in normal man. The striking advantages offered by such patients are that first, by the simple expedient of clamping their Torkildsen bypassing tubes, the main ventricular compartments are effectively isolated from the subarachnoid space, and any communication between them by direct flow is precluded. Thorough mixing within each compartment can then be achieved by barbotage, and the volume of the compartments readily measured. Turnover constants calculated from the data are characteristic of the entire compartment, and when corrected for the volume of the compartment may be meaningfully compared. Finally, isolation of the subarachnoid space from the alleged main fountainhead of CSF, the choroid plexuses of the first three ventricles, provides a critical test of the importance of these structures in supplying the CSF of the subarachnoid space, or alternatively, evidence for the existence of some other important source.

Our experimental technic consisted of fixing small bore needles in the lateral ventricles and lumbar subarachnoid space, leaving them in position for the duration of the study, to obviate leakage from the puncture sites. The isotopic solutions were prepared in small volumes with physiologically insignificant amounts of carrier, and were injected either intravascularly or directly into the CSF system. After thorough mixing of the compartment, samples were withdrawn in less than 0.5 cc quantities, and metered out with calibrated Kinsey pipettes. The radioactivity of these CSF samples and of plasma samples was assayed in a well-type scintillation counter, and compared with a standard prepared in known dilution from an aliquot of the injection solution. K-42 and Cl-38 were chosen as being the most expedient isotopes for studying simultaneously major intracellular and extracellular ions. The 38 minute half-life of Cl-38 and 12.4 hour half-life of K-42 are sufficiently diverse to permit the assay of both isotopes from a single sample by serial counting before and after the chloride has decayed to inappreciable levels. We have measured ventricular volumes by isotopic dilution methods and checked these by dye dilution using indigo carmine and by planigraphic measurement of the ventricles on X-rays taken during pneumoventriculography. The volume of the subarachnoid space was measured in patient S.P. by the dilution of a known activity of I^{131} - human serum albumin immediately after protracted barbotage. Because of the short life of Cl-38, it was necessary to carry out much of this work at Brookhaven National Laboratory.‡ where Cl-38 is produced in the atomic pile.

Results. A. *Electrolytes and water.* Fig. 1 depicts the type of CSF appearance curves

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‡ We are greatly indebted to Dr. Lee Farr, Director of the Medical Department, and his associates Drs. Dahl, Robertson, and Stickley, for their extraordinary cooperation and generosity in aiding the performance of these studies.

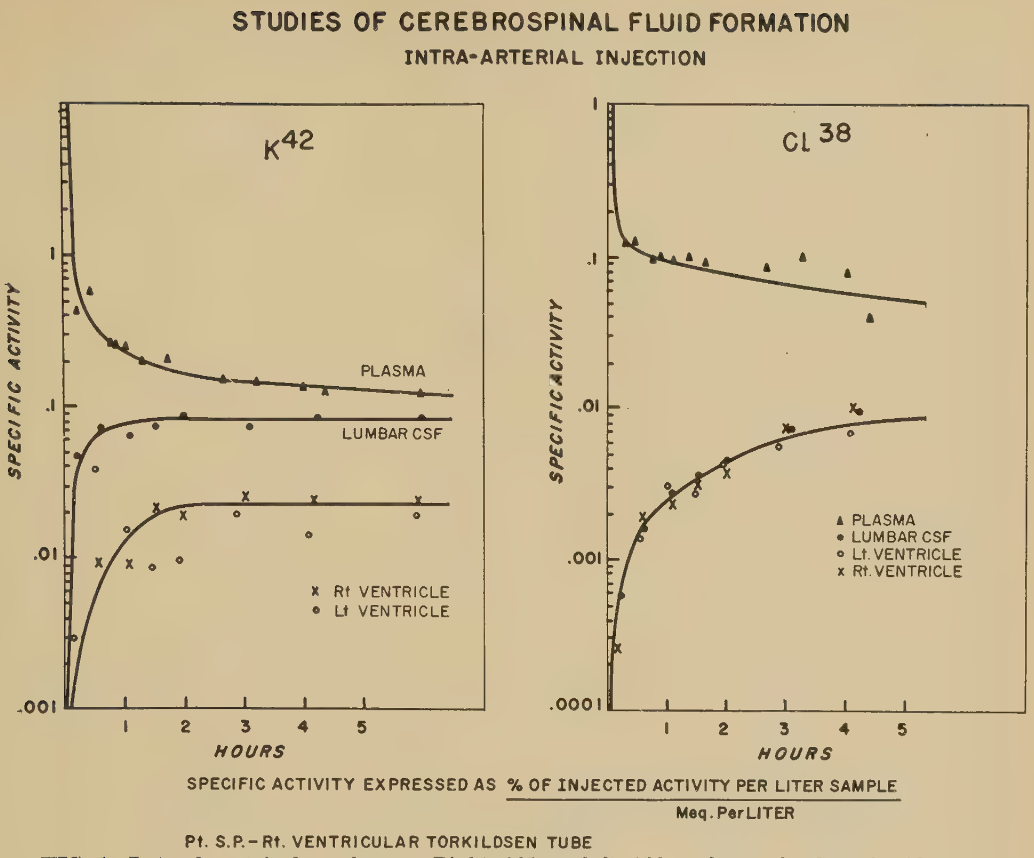
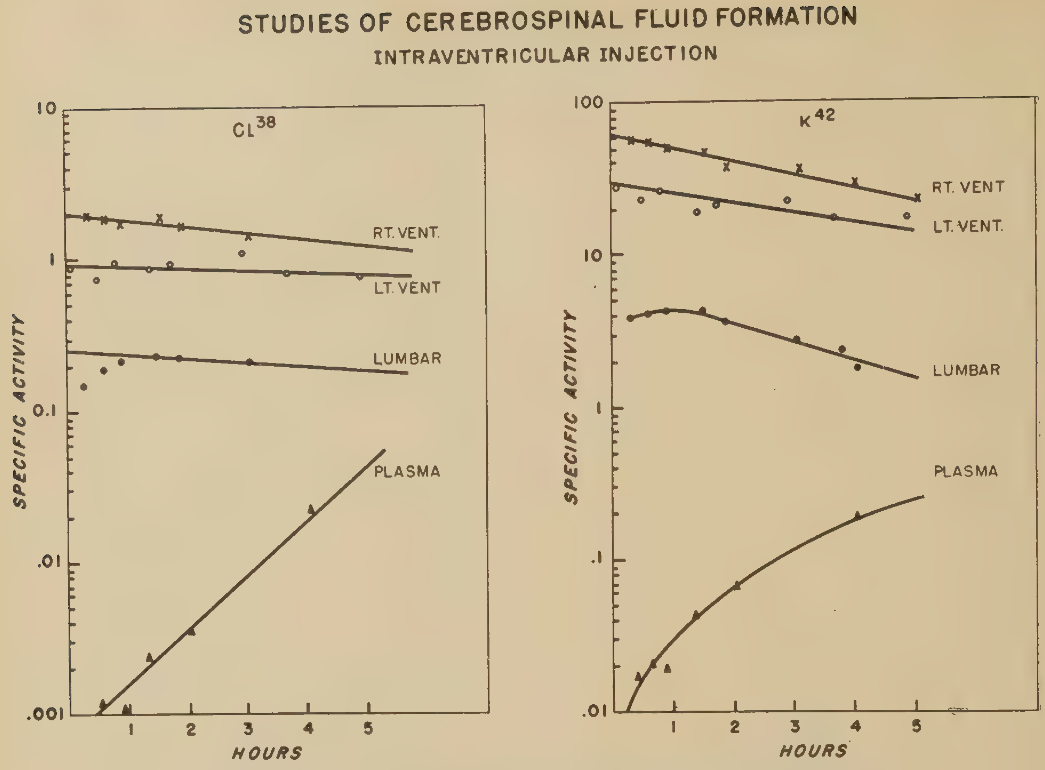


FIG. 1. Lateral ventricular volumes: Right, 301 cc, left, 320; volume of subarachnoid space: 83 cc.

obtained after intravascular injection of K-42 and Cl-38 with the ventricular compartments isolated from the subarachnoid space. One sees that in the case of potassium there is actually a far more rapid rise in isotopic concentration in the lumbar CSF than in the ventricles, and that chloride appears at the same rate in all 3 sites. We consider this to be decisive evidence for a rapid and direct exchange of electrolytes between blood and the subarachnoid space which is independent of flow from the choroid plexus. That a similar situation exists for water we have demonstrated in a previous communication (5). In this, the rate of appearance of D_2O was measured at various points in the CSF system of patients entirely normal in this respect following intravenous injection. D_2O in the cisternal CSF was seen to reach equilibrium much more rapidly than it did in the ventricles—an impossible situation if cisternal fluid is assumed to be derived from the ventricles by flow.

That reabsorption of water and electrolytes also occurs throughout the system we have demonstrated by studies of the disappearance of isotopes injected into the isolated ventricular compartments and into the subarachnoid space. The results of such a study employing again K-42 and Cl-38 are shown in Fig. 2. It may be seen that both of these substances pass rapidly out of the ventricles according to a simple exponential function, with turnover half-times of 4.3 hours and 8.2 hours for K and Cl respectively. The only possible routes of egress here are a retrograde passage through the choroid plexus supplemented by diffusion through the ependymal wall into the brain. D_2O injected into the ventricles of this patient at the same time as the electrolytes behaved


STUDIES OF CEREBROSPINAL FLUID FORMATION
INTRAVENTRICULAR INJECTION

SPECIFIC ACTIVITY EXPRESSED AS $\frac{\text{\% OF INJECTED ACTIVITY PER LITER SAMPLE}}{\text{Meq. Per LITER}}$

Pt. S.L. – LEFT VENTRICULAR TORKILDSEN TUBE

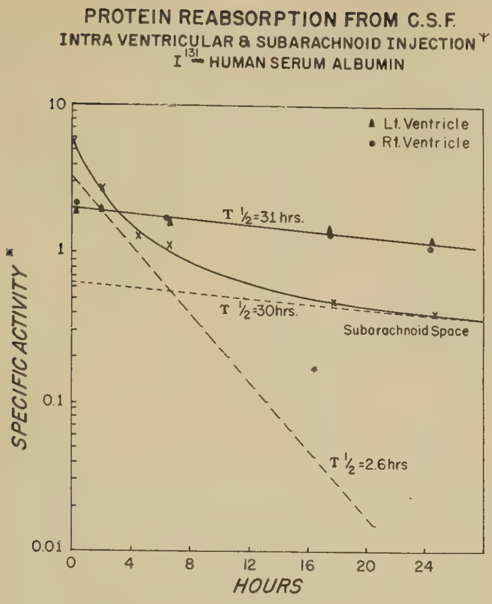
FIG. 2. Ventricular volumes: Right 220 cc, left 220 cc. The intraventricular inj. in this patient was carried out with the Torkildsen tube open. This permitted a small quantity of the isotopes to pass into the subarachnoid space; hence the rise during the first hour of isotopic concentrations in the lumbar region. The tube was closed during the last 4½ hr of study.

similarly with an average turnover half-time for ventricular water of 40 minutes and in subarachnoid space of 72 minutes.

A third feature of the behavior of electrolytes is that they move in and out of the CSF independently and each at its own characteristic rate. This is evident in the foregoing data and is supported by other multiple tracer studies done with various combinations of Na-24, Br-86, Cl-38 and P-32 in other patients.

It is thus seen that the water and electrolytes of CSF are in dynamic equilibrium with plasma, and that CSF is renewed by local exchange therewith in every region. Hence any net increase in volume within any compartment capable of initiating flow must be dependent either on a small secretion pressure (active transport) or on some alteration in the balance of hydrostatic and osmotic forces.

B. *Protein.* In clinical or experimental (Dandy(2)) instances where outflow from the ventricles is blocked for any protracted period of time, a slight unbalance in the equilibrium condition appears actually to occur since over a period of days or weeks increased pressure and an expanded ventricular volume results—clinically known as hydrocephalus. By analogy to the situation in the systemic circulation where the constant sweeping away of protein by the lymphatics is essential to the balance of transcapillary exchange, it occurred to us that a slow rate of flow away from the ventricles is necessary to prevent the accumulation of protein and the resulting shift in equilibrium due to the increased osmotic tension. In order to test this hypothesis, we have studied the rate of reabsorption of



PROTEIN REABSORPTION FROM C.S.F.
INTRA VENTRICULAR & SUBARACHNOID INJECTION[¥] OF I^{131} — HUMAN SERUM ALBUMIN

[*] ACTIVITY EXPRESSED AS % OF TOTAL COMPARTMENT DOSE PER Mg. CSF PROTEIN

[¥] DOSE: 24μC (0.5mg. HSA) PER VENTRICLE, 12μC → SUBARACHNOID SPACE

Pt. S.P - Rt. VENTRICULAR TORKILDSEN TUBE

FIG. 3.

radio-iodinated human serum albumin from the isolated ventricles and subarachnoid space. Fig. 3 illustrates one set of results. Albumin was reabsorbed from the ventricles very slowly as a simple exponential function with a turnover half-time in this patient, of 31 hours. In sharp contrast to this, the rate of disappearance from the subarachnoid space was considerably more rapid and could be resolved into two exponential components: one with a half-time of 30 hours, essentially the same as the ventricular rate, and the other with a turnover half-time of 2.6 hours. This provides direct evidence for our theory that in contrast to the water and electrolytes of the CSF, protein is absorbed largely from the sub-arachnoid space. Based on abundant evidence in the older literature that the integrity of the arachnoidal villi is essential to the prevention of hydrocephalus (Weed(3)) and on demonstrations that certain large negatively charged molecules such as gelatin (Key and Retzius(6)) and ferrocyanide (Weed (7,8)) are specifically absorbed by the arachnoidal villi, it is our belief that these specialized structures subserve the special and essential function of protein reabsorption from the CSF. In this function, the arachnoidal villi become analogous to the lymphatics of the general circulation. To imbue them however, with the entire task of CSF absorption as in traditional theory is just as unsound as it would be to ascribe the entire return of capillary filtration to the lymphatics.[§]

There is a corollary to the facts that electrolytes and water of the CSF exchange everywhere freely whereas protein is absorbed largely from the subarachnoid space—namely that the site and rate of protein reabsorption is an important determinant of the direction and rate of flow of CSF within the system. We have attempted to determine the order of magnitude of net CSF formation and flow from the ventricles in these patients whose by-passing tubes from the lateral ventricles to the cisterna magna we could block. During the study, the patient was kept in a semi-reclining position at an angle such that the initial intraventricular pressure within the tube blocked was only 10- 20 mm above the vertex of the head. Over the 24 hour period of the study, withdrawal of 0.35 cc samples at less than hourly intervals for the assay of isotopic concentrations sufficed to keep the CSF pressure virtually constant. This was measured periodically, without withdrawal of fluid, between the times of sampling. At the conclusion of the 24 hours in one patient, the final pressure was a few millimeters of water lower than the initial pressure during which time a total of about 15 cc of fluid had been withdrawn and the patient's clinical condition and arterial blood pressure had remained in the same excellent state. Since at the end of the study the intraventricular pressure was slightly lower than initially, it seems improbable that any increase in ventricular volume occurred. Thus our figure of 15 cc withdrawn over the 24 hours must represent a fair estimate of the net amount of CSF elaborated in the lateral ventricles of this patient per day. This estimate is substantially lower than the previous figures given in the literature for man: 300 to 400 cc/day by Dandy(2), and 600-700 cc/day given by Boyd(9). It is also proportionately lower considering the size of the species, than Flexner's(10) figure, for the cat of 12 cc/day flowing from cannulas in the aqueduct of Sylvius.

[§] Professor A. Baird Hastings has repeatedly emphasized to us his opinion that the CSF might well turn out to be "the lymphatic fluid of the central nervous system". We are indebted to him for much aid in this work.

Summary. 1. Water and electrolytes enter the cerebrospinal fluid rapidly both in the ventricles and throughout the subarachnoid space. The formation of the fluid is thus not an exclusive prerogative of the choroid plexus. 2. Water and electrolytes leave directly into the blood from both the ventricles and the subarachnoid space, doing so at roughly similar rates. 3. CSF does not suddenly come into being fully formed at any point with all its constituents in their proper ratios, but each constituent is exchanging with blood at its own characteristic rate. 4. In contrast to water and electrolytes, protein is absorbed largely from the subarachnoid space, presumably from the arachnoidal villi. By virtue of this specific role played by the arachnoid villi, they serve a function in the CSF system analogous to the lymphatics of the general circulation. 5. The site and rate of protein reabsorption is an important determinant of the direction and rate of CSF flow. 6. The net amount of CSF elaborated per day in the ventricles, over and above the large and equivalent-volume exchanged, is small, and is of the order of 10 to 20 cc or less, per day in man.

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Received October 14, 1953. P.S.E.B.M., 1953, v84.

Natural Abundance of N^{15} in Hemin and Plasma Protein from Normal Blood.* (20660)

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Geren, Bendich, Bodansky and Brown(1) found the abundance of N^{15} in nitrogen of biological origin to be slightly higher than that of tank nitrogen used as a reference standard in the case of urinary urea, ammonia and uric acid. Urea isolated from the urine of normal individuals analysed 0.0044 ± 0.0005 atom per cent higher than tank nitrogen, and the total nitrogen, ammonia and uric acid from normal urines had N^{15} concentrations of about the same magnitude.

In connection with studies of erythropoiesis in the anemia of thermal injury(2) and of the metabolic fate of N^{15}-labelled erythrocytes transfused into normal man(3), we have had occasion to determine N^{15} abundance in a number of samples of hemin and plasma protein isolated from blood before administration of N^{15}-glycine. We have observed in hemin and plasma protein an increased abundance of N^{15} of the same magnitude found in compounds of urinary origin. For calculation of the amount of N^{15} incorporated from N^{15}-

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* Studied in connection with projects conducted under contract between the Office of the Surgeon General, Department of the Army, Washington, D.C. and the Medical College of Virginia and by a grant from the U. S. Public Health Service to Dr. E. I. Evans, Director of the Laboratory for Surgical Research, Medical College of Virginia.

TABLE I. N^{15} Concentration in Hemin and Plasma Protein Isolated from Normal Blood.

	Hemin		Plasma protein
	10 dogs	9 humans	7 humans
	Atom % excess		
	.0055	.006	.004
	.004	.006	.0035
	.0055	.004	.004
	.0055	.006	.004
	.005	.0035	.004
	.003	.003	.004
	.0055	.0025	.0035
	.0045	.004	
	.004	.005	
	.0045		
Avg	.0047	.0044	.0039
S.D.	.0009	.0014	.0003

glycine into heme of the total circulating hemoglobin in different subjects for comparative purposes, correction for the slight excess of N^{15} normally present in heme may become appreciable, especially at the lower N^{15} concentrations encountered in the hemin isolated after N^{15}-glycine feeding during depressed erythropoiesis(2).

Methods. Hemin was isolated as described by Shemin and Rittenberg(4) and recrystallized according to Fischer(5). Plasma proteins were precipitated and washed 5 times with trichloroacetic acid(6), twice with acetone-ether (1:1), once with ether and dried. Duplicate samples were analyzed for N^{15} in a Nier-type isotope-ratio mass spectrometer which was built and maintained by Dr. William T. Ham, Jr., Mr. Ray Williams, and Mr. Fred Schmidt of the Biophysics Department of this institution. Each of the duplicate samples was compared with tank nitrogen as a reference standard and the N^{15} atom per cent excess over tank nitrogen determined. N^{15} atom percentages were rounded off to the third decimal place from the calculated values (7).

Results. In Table I are presented the results of analyses of 19 hemin samples analyzed along with other samples by our usual procedures at different times over a period of more than a year. The values presented are the averages of duplicate determinations which usually did not differ by more than 0.001 atom % excess. The mean values for dog hemin: 0.0047, human hemin: 0.0044, and for the 19 hemin samples combined: 0.0046 ± 0.0011 atom % excess, are very close to those found with urinary urea(1).

The different samples of normal human plasma protein were analyzed on the same day and hence the variation among samples was much smaller than in the case of the hemin samples which were determined under varying conditions. The value 0.0039 ± 0.0003 atom % excess N^{15} also shows a slightly increased abundance over tank nitrogen of a similar magnitude.

Sixteen samples of human plasma protein isolated and analysed over a 5-month period showed a wider variation among samples (mean value 0.0036 ± 0.0015 atom % excess). Although the subject in this case had received an infusion of about 500 ml of washed N^{15}-labelled red cells(3), this apparently did not affect the N^{15} concentration in the plasma proteins.

No difference from tank nitrogen was noted with nitrogen from ammonium chloride, the mean difference of 6 samples being less than 0.0001 ± 0.0007 atom % N^{15}. Fractionation of the tank nitrogen used as a reference standard was therefore not responsible for the results obtained with the biological samples. Soloway(8) also found no detectable fractionation of tank nitrogen when compared with that of ammonium sulfate or of air. Our results thus appear to lend further support to the idea that minute fractionation of the nitrogen isotopes might occur through normal biological processes(9).

Summary. Hemin and plasma protein isolated from normal blood were found to have a slightly increased abundance of N^{15} over tank nitrogen or ammonium chloride. The mean values for normal hemin, 0.0046, and for normal plasma protein, 0.0039 atom % excess N^{15}, were similar to the N^{15} concentration found in urinary urea. These results lend further support to the idea that minute fractionation of the nitrogen isotopes might occur through normal biological processes.

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Received October 14, 1953. P.S.E.B.M., 1953, v84.

Influence of pH on Toxicity of Vanadium in Mice. (20661)

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In testing various compounds as antidotes of vanadium(1,2), it was found that one of the substances (ascorbic acid) that afforded good protection against vanadium toxicity had a pH of 2.5 under the conditions of use. This suggested the possibility that H-ion concentration might alter toxic response to vanadium. Support for this possibility was found in a report that anionic vanadium is more toxic than cationic vanadium(3). Moreover, Aub *et al.*(4) showed long ago, and it has been amply confirmed since, that urinary lead excretion is increased following administration of ammonium chloride. More recently, Brown, Kolmer, and Rule(5) showed a similar effect for bismuth. It has further been postulated that an important factor in detoxication is the conversion of a weakly acidic substance, which the body excretes with difficulty, to a relatively strong acid which can be readily eliminated by the kidney(6). Accordingly, test were performed on mice to determine whether altering the pH in various ways would affect vanadium toxicity.

Materials and methods. Male mice of the Webster strain* weighing 20-29 g were used for this study. Each mouse was weighed individually and injected with the agents in doses according to body weight. The intraperitoneal route was used for all injections, since more consistent responses could be obtained by this route than by oral or subcutaneous administration. The mice were injected with substances suspected of altering vanadium toxicity into the left side of the peritoneal cavity, followed in 15 minutes by sodium metavanadate ($NaVO_3 \cdot H_2O$) injected into the right side of the peritoneal cavity. Solutions of sodium metavanadate were prepared in physiologic saline solution at a concentration of 4 mg/ml with a resultant pH of 7.0.

TABLE I. Effect of Administering Ammonium Chloride and Bicarbonate, 15 Minutes Prior to Vanadium, on Vanadium Toxicity in Mice. Fatality rate expressed as ratio of No. of animals that died (numerator) to those used (denominator).

mg/kg $NaVO_3 \cdot H_2O$	mg/kg as V	Controls, no prior treatment	NH_4Cl, 400 mg/kg	$NaHCO_3$, 2 g/kg
25	9.1	8/20		17/20
30	10.9	12/20	5/20	

Results. Table I shows that ammonium chloride administered 15 minutes prior to vanadium resulted in a decrease of toxicity of $NaVO_3 \cdot H_2O$ at a level of 30 mg/kg. The mortality ratio of 12/20 (60%) was reduced to 5/20 (25%) by the acid chloride. Bicarbonate administered in the same manner conversely increased the toxicity of 25 mg/kg $NaVO_3 \cdot H_2O$ from 8/20 fatalities (40%) to 17/20 (85%). The doses of ammonium chloride and sodium bicarbonate used in the above tests, were the largest doses which could be used without producing signs of toxicity in the mice as determined by separate toxicity tests.

Table II shows that acidifying the $NaVO_3 \cdot H_2O$ solution to pH 1.8 before in-

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* Mice were obtained from Hamilton Laboratory Animals, Inc., Cincinnati, O.

TABLE II. Toxicity of Acid and Alkaline Solutions of $NaVO_3 \cdot H_2O$ and of a More Acidic Vanadium Salt. Fatality rate expressed as ratio of No. of animals that died (numerator) to those used (denominator).

mg/kg as V	$NaVO_3 \cdot H_2O$ solutions pH 7.0	pH 1.8	pH 12.5	NH_4VO_3, pH 6.1
9.1	8/20		7/20	
10.9	12/20	1/20		2/20
14.6	17/20	0/20		14/20

jection resulted in decreasing the toxicity of 10.9 mg/kg V (LD60)† and 14.6 mg/kg V (LD85)† doses to the point that these doses were practically nonlethal. Alkalinization of the vanadium solution prior to injection as seen in Table II had no effect on $NaVO_3 \cdot H_2O$ toxicity. As can be seen also in Table II, column 5, NH_4VO_3 is only slightly less toxic than $NaVO_3 \cdot H_2O$ at 14.6 mg/kg V; however, when the two compounds are compared at 10.9 mg/kg V, NH_4VO_3 is found to be lethal to 10% of the mice, whereas, $NaVO_3 \cdot H_2O$ is lethal to 60% of the mice. The solutions of $NaVO_3 \cdot H_2O$ and NH_4VO_3 used in this comparison were made to the same concentration in respect to vanadium and were not buffered.

Discussion. From the data presented, it appears that detoxication of vanadium may be affected by the bicarbonate reserve of the blood. Since alkalinization of a vanadium solution did not increase the toxicity, whereas injecting large amounts of bicarbonate into the mice did result in an increase in vanadium toxicity, it appears that excessive blood bicarbonate may retard the process of converting vanadium to a less toxic state. One of the means by which vanadium is detoxified appears to be acidification. This is further supported by the fact that ammonium chloride administered prior to vanadium decreased vanadium toxicity and acidification of vanadium solutions prior to injection, markedly reduced the toxicity.

On the basis of the foregoing results, it was predicted prior to testing that ammonium metavanadate would be less toxic than sodium metavanadate for 2 reasons: 1) Ammonium vanadate is more acid than sodium vanadate, and 2) it is also likely that NH_3 would be metabolized by the body to form urea, leaving HVO_3 a stronger acid as residue.

Summary. 1. Data have been presented to show that pH plays an important role in the toxic response of mice injected with vanadium. Shifting the pH of vanadium solutions toward greater acidity tended to decrease the toxicity. 2. One of the processes by which vanadium is detoxified is probably acidification. Increasing the blood bicarbonate conversely appears to retard detoxication.

Acknowledgment is herewith made to Dr. Herbert E. Stokinger for helpful suggestions and review of this manuscript.

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Received October 14, 1953. P.S.E.B.M., 1953, v84.

Breakdown of Urea in the Rat. (20662)

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At the present time there is an apparent conflict in the evidence regarding the stability of urea in the animal organism, especially in the rat. Bloch(1) found that when urea-N^{15} was fed to rats over a period of 3 days the N^{15} concentration in the excreted ammonia was only 4% and that in the liver protein was less than 2% of that in the urinary urea. This led him to believe that urea once formed in the rat was subject to little breakdown. The more recent experiments of Kornberg and Davies(2), carried out in the cat led to quite similar results. In contrast to these results obtained with urea-N^{15}, Leifer and coworkers (3) found that when urea-C^{14} was administered to mice 21% of the C^{14} appeared in the respiratory air in 48 hours. Skipper and coworkers(4) found that in the same species about 9% appeared in the respiratory air in 10 hours and a total of 80% of the C^{14} was recovered in the urine after 24 hours. These data indicate a great lability of urea in the mammal and are in agreement with the nutritional data of Lardy and Feldott(5) and Rose and coworkers(6) who have shown that the growth of rats, on diets containing nitrogen in the form of essential amino acids only, could be improved by adding urea or ammonium salts to the diet. Since urease is present in the gastric mucosa of most species(7-9), it is tempting to attribute the breakdown of urea which has been observed, to the action of this enzyme. However, it is equally possible that in the intact animal the major part of the decomposition is due to the action of the intestinal microflora.

TABLE I. Metabolism of Urea-C^{14} in Rats. Experiments were on normal rats except Ip.3 in which the animal was pretreated with succinyl sulfathiazole for 2 days and in Sc.2 in which the animal was eviscerated. The experimental period was 24 hr except in the eviscerated animal in which the period was 9 hr.

			Fraction of dose excreted in exp. period	
Exp.*	Wt of rat, g	Dose of urea,† mg	Urine, %	Respired air, %
Ip.1	230	4.5	43.8	40.4
2	250	4.5	67.5	30.5
3	244	4.75	69.5	18.9
Sc.1	240	4.75	75.6	21.2
2	250	2.5	24.9	3.6

* Ip. and Sc. indicate inj. of urea made into the peritoneal cavity or subcut.

† In terms of radioactivity dose varied between 1.4 and 2.8 × 10^6 counts/min.

The object of the present experiments was to determine the site of urea breakdown in the intact rat and to assess its significance. The results show that urea-C^{14} when injected into normal rats is decomposed to a considerable extent into carbon dioxide, up to 40% appearing in the respiratory gases in the first 24 hours. The eviscerated rat, however, breaks down urea at a comparatively slow rate. Isolated tissues from rats were also found to cause comparatively little urea breakdown, although the reverse was the case with washings from the intestine. It is clear from these experiments and from those of Bloch, who used urea-N^{15}, that in the rat, urea breakdown occurs continually at a rather rapid rate due to the activities of urease in the intestinal flora and gastric mucosa. The ammonia is again reconverted into urea. This conversion has been repeatedly demonstrated both *in vivo* (10) and by enzymatic experiments.

Methods. The urea-C^{14} was purchased from the U. S. Atomic Energy Commission to whom our thanks are due. Before use it was diluted with suitable amounts of inert urea. The Long-Evans rats used in the *in vivo* experiments were adults with weights shown in Table I. They were maintained on a diet of Purina laboratory chow prior to the experiment. After the injection of the urea, which was made either subcutaneously or into the peritoneal cavity, the animals were kept in an all glass metabolic cage through which a slow stream

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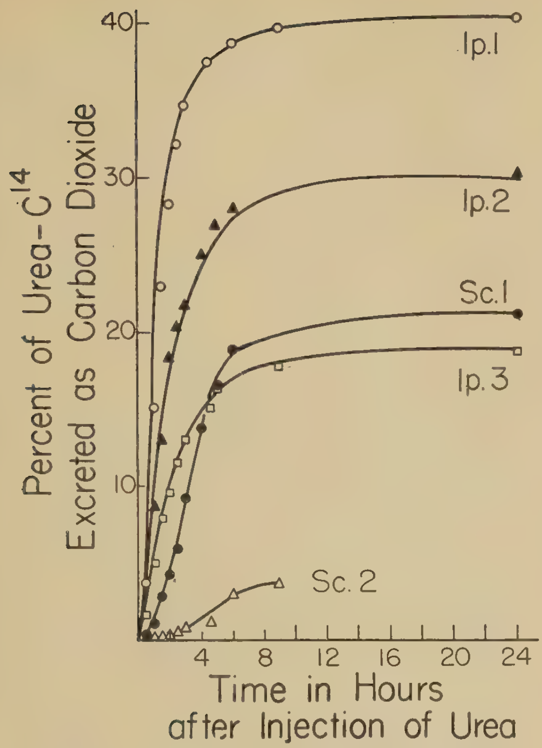

FIG. 1. Cumulative excretion of carbon dioxide-C^{14} following the administration of urea-C^{14} to rats either into the peritoneal cavity (Ip.) or subcutaneously (Sc.). In exp. Ip.3 the animal was treated with succinyl sulfathiazole, and in Sc.2 an eviscerated animal was used.

of carbon dioxide-free air was passed. The expired carbon dioxide was collected in sodium hydroxide using 2 milli-equivalents of the base per minute of collection time. Urine was collected in dilute hydrochloric acid contained in flasks connected to the metabolic apparatus. The rat used in experiment Ip. 3 was pretreated with succinyl sulfathiazole for two days (1 ml 10% solution twice a day) prior to the administration of the urea, and that used in experiment Sc. 2 was eviscerated following an overnight fast. Our thanks are due to Dr. W. O. Reinhardt for performing the operation. Rat tissues and washings from the intestine used in the *in vitro* experiments were incubated in flasks with an inlet tube equipped with a sintered glass tip. The outlet tubes were provided with absorbers for carbon dioxide which was swept over with a slow stream of oxygen. For medium, Krebs-Ringer-phosphate buffer containing 0.4 g glucose per 100 ml was employed at a temperature of 38°. For the determination of radioactivity the carbon dioxide was precipitated as the barium salt which was washed, dried and then sedimented on to aluminum discs. Aliquots of urine were dried on glass discs at room temperature. All radio activities were measured in duplicate on a Geiger-Müller counter, and were corrected for self-absorption.

Results and discussion. The extent to which the C^{14} from urea was excreted in the urine and expired air by rats under several conditions is shown in Table I, and the time course of the appearance of the C^{14} in the carbon dioxide is shown in Fig. 1. From these data it is clear that there is a considerable and quite rapid decomposition of urea to give carbon dioxide in the intact rat, and that the amount decomposed is less when the substance is given subcutaneously. It is also evident that the decomposition was reduced by pretreating the animal with succinyl sulfathiazole, and

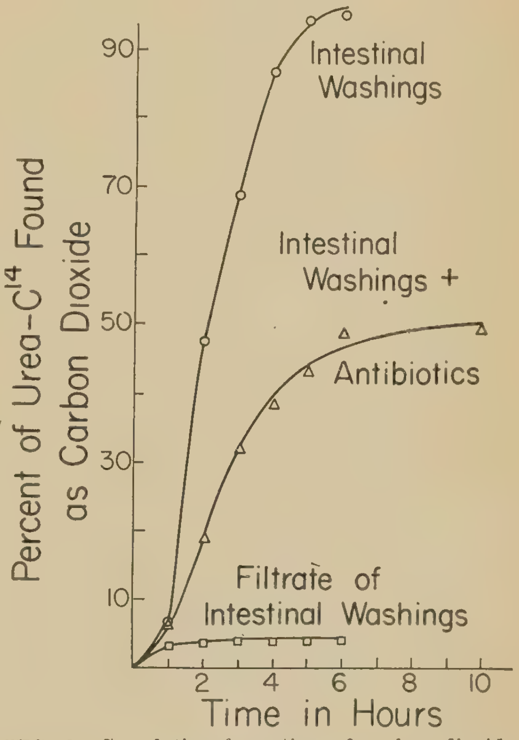

FIG. 2. Cumulative formation of carbon dioxide from urea-C^{14} by washings from the intestine. Antibiotics used/10 ml, penicillin 50000 units, dihydroxystreptomycin 2 mg, sulfasuxidine 4 mg, sulfathiazin 1 mg.

TABLE II. Metabolism of Urea in Isolated Tissues from the Rat.

About 1 g wet wt of tissue was incubated in Krebs-Ringer-Phosphate containing 0.4% glucose and 1 mg of urea-C^{14}, 3.2 to 5.6 × 10^5 c.p.m. and the CO_2 formed was aerated into base. Samples were taken at hourly intervals.

	Fraction of urea-C^{14} appearing as CO_2		
Interval, hr	Liver slices, %	Kidney slices, %	Stomach sections, %
0-1	.15	.16	.04
0-2	.23	.19	.09
0-3	.24	.23	.14
0-4	.24	.41	.16
0-5	—	—	.20
0-6	—	.49	.30

especially by evisceration. In the last mentioned condition there was little appreciable decomposition of the urea at all, particularly in the first 3 or 4 hours of the experiment. Subsequently, the rate appeared to increase which may be due to contamination with bacteria.

The production of respiratory carbon dioxide follows monomolecular reaction kinetics from 0.5 to about 5 hours after the injection of the urea when the substance is given intraperitoneally, the half time being of the order of one hour. Gould and coworkers(11) found a half-time for loss of carbon dioxide following the injection of bicarbonate to be about 15 minutes. Hence, the rate governing process must be that for urea decomposition.

The *in vitro* experiments with liver, kidney and stomach tissue show that there is little decomposition of urea by these tissues(Table II). It is evident that these organs could not account for the extensive urea breakdown observed in the *in vivo* experiments. However, when intestinal washings were incubated with urea considerable decomposition occurred in a few hours. This decomposition was not found when the washings were filtered, nor to such a great extent when antibiotics were present. Taken together with the previous results it appears that the greater part of the urea breakdown must be attributed to the activity of the intestinal flora and not to the tissues of the rat itself.

The indications from these experiments are that urea is not a stable substance in the rat but rather that it undergoes a continual decomposition. There is a type of urea cycle in the animal. Urea being freely diffusible appears in the secretions into the intestine and is in part decomposed there by the microorganisms. The resorbed ammonia is immediately reconverted to urea by the liver.

Sprinson and Rittenberg(12) and others (13) have administered amino acids such as glycine-N^{15} to animals in attempts to deduce rates of turnover of body protein from the rate of excretion of the N^{15}, largely in urea. It is obvious that the urea breakdown-resynthesis mechanism which has been shown to exist may modify the N^{15} excretion rate, and that in animals under different conditions, with different intestinal flora, different rates of N^{15} excretion may exist for this reason alone.

Summary. A rapid breakdown of urea-C^{14} is observed in the intact rat but this is entirely absent in the eviscerated animal. Due to the existence of this breakdown which is immediately followed by resynthesis, there is a type of urea cycle in the intact animal. No significant breakdown of urea occurs in either liver, kidney or stomach, but the intestinal washings rapidly decompose the substance.

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Received October 14, 1953. P.S.E.B.M., 1953, v84.

Resistance in Leukemic Cells to an Adenine Antagonist, 6-Mercaptopurine. (20663)

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Transformations have been obtained to resistance or dependence in leukemic cells of the mouse by the use of several antimetabolites: folic acid analogs(1,2-4) and the triazolopyrimidine analog of guanine, 8-Azaguanine(5). Such transformations have been shown to be stable, irreversible and heritable, and have been produced readily in several transplantable lines of lymphocytic leukemia (6). Experimental findings favor the assumption that the variant lines produced by the use of antimetabolites arise by mutation and the role of antimetabolite is merely as a selective agent(7).

Preliminary evidence which suggests that purine analogs act as antagonists of nucleic acid synthesis has encouraged investigation of the role of these compounds in the inhibition of cancerous growths. The adenine analog, 6-mercaptopurine, has been shown to act as a purine antagonist in the metabolism of *Lactobacillus casei*(8). It has also been shown to be a unique inhibitor of Sarcoma 180(9) and of certain mammary adenocarcinomas(10). Limited clinical trials of this compound in advanced leukemia of children have been encouraging(11).

Definite, regular and reproducible inhibition of growth of leukemic cells has been obtained in 2 transplantable lines of acute lymphocytic leukemia. No increase in survival time of mice bearing 2 other transplantable acute lymphocytic leukemias could be obtained. In leukemia L 1210(12), particularly, striking increases in survival time were observed and are described here. Following the methods used previously in obtaining transformations to folic acid antagonists(4) and 8-Azaguanine(5), a resistant subline of leukemia L 1210 has been developed using 6-mercaptopurine as the selective agent. Some of the pertinent characteristics of these L 1210 resistant leukemic cells will be considered.

Transplant generations 202 to 251 of the sensitive subline of leukemia L 1210 have been used as control material for the present study. Beginning with the 202nd transfer, a standard dose of leukemic cells (8×10^5 cells) in Lockes' solution was inoculated subcutaneously in the right axillary region of 18-20 g DBA/2 strain mice. These mice subsequently received daily injections, beginning at 24 hours, of the adenine analog, 6-mercaptopurine.[†] The compound was dissolved in 0.1 N NaOH then diluted with sterile saline so that the constant volume injected subcutaneously was 0.025 cc per g of body weight. Daily injections were continued until palpable growths sufficient for transfer were obtained. During the first 3 transfers the total dosage of analog was 262.5 mg/K (37.5 mg/K x 7 days) and subsequently the total dosage was 525 mg/K (75 mg/K x 7). Transfers of leukemic cells were done at 9 to 11 days during the first 4 transfer generations and later at 7 and 8 days as larger subcutane-

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[*] National Institutes of Health, U. S. Public Health Service.

[†] This compound furnished by Dr. George Hitchings of The Wellcome Research Laboratories, Tuckahoe, N. Y. A-methopterin and 8-Azaguanine, were supplied by Lederle Laboratories Division, American Cyanamid Co.

TABLE I. Development of Resistance in Leukemia L 1210 Following Continuous Treatment with Adenine Analog 6-Mercaptopurine. DBA strain of mice.

Transfer generations	No. mice	Time transplanted, days	Dosage (mg/kg) Daily	Total	Tumor wt (mg) at 8 days
L 1210-6-mercaptopurine-resistant					
G- 1 to 3‡	3*	9–11	37.5	262.5	0
4	5	8	75	525	280.4
4	5	8	0		420
5 to 7	3*	8	75	525	†
8	5	8	75	525	81
8	5	8	0		45
9 to 12	3*	8	75	525	†
13	10	8	75	525	200
13	10	8	0		251.8
14 to 39	5*	7–8	75	525	Approx. 500
L 1210 sensitive					
206	8	8	75	525	0
	8	8	0		450.9
210	8	8	75	525	0
	8	8	0		496.3
212	8	8	75	525	0
	8	8	0		590.2

* Each.

† Carried only.

‡ Transfer generation G-1 was derived from transfer generation G-202 of the L 1210 sensitive line; thus, transfer generation G-206 is the control transfer for G-4 of the resistant subline.

ous growths appeared in animals receiving the near-MTD dosage. The difference in response to 6-mercaptopurine in the transfer line grown only in mice receiving the analog and in the control (sensitive) line may be seen in Table I. Very little difference in weights of the subcutaneous tumor grown in mice with and without analog were observed in the line being developed for resistance whereas complete inhibition of growth of leukemic cells, at 8 days, was observed in the sensitive control line at comparable transfer generations.

The 6-mercaptopurine-resistant subline has now been carried through 39 consecutive subcutaneous transfers in DBA/2 mice receiving the near-MTD of this analog, 525 mg/K (75 mg/K x 7). Sizeable tumor growths of nearly 500 mg are always obtained in the presence or absence of the analog, indicating the development of resistance. The dependence phenomenon characteristic of other transfer lines has not been observed although only one other variant subline (also resistant) has been developed in this laboratory using this compound. That the variant cells described here exhibit resistance to 6-mercaptopurine is shown also in experiments using leukemic death, following transfers intraperitoneally, of the standard dose of leukemic cells, as the criterion. The mean survival time of mice bearing the sensitive subline of L 1210 was 7.9 ± 0.06 days whereas an increase of survival of 87.3%, to 14.8 ± 0.18 days was obtained using 6-mercaptopurine within the total dosage range of 250-1200 mg/K. In contrast, identical survival times were obtained in mice bearing the 6-mercaptopurine-resistant cells, with and without the analog (Table II).

Significant and reproducible increases in survival time in DBA/2 or CDF_1 mice bearing the sensitive, control leukemia L 1210 were obtained at dosage levels of 250 mg/K (50 mg/K x 5 days) to 1000 mg/K (125 mg/K x 8 days). Slight losses in body weight, never exceeding 1.5 g, were obtained only at dosage levels of 600-1000 mg/K. Within the dosage range of 250 to 600 mg/K, regular increases in body weight were obtained. No deaths attributable to the drug were found at the highest dosage level used. The effects obtained at the higher dosage levels were within the same range as those obtained at the 300-600 mg/K level.

The possibility was considered that resistance developed to one purine-antagonist might extend to other purine antagonists and possibly folic acid antagonists. Of the compounds used, the triazolopyrimidine analog, 8-Azaguanine has been shown to have leukemia-inhibiting properties in certain leukemias, especially in the lymphocytic leukemia L 1210 used in this study. Thioguanine also has been shown to effectively and regularly increase survival time of L 1210 leukemic mice whereas 8-Azaxanthine (resulting from deamination of 8-Azaguanine) and 2,6-diaminopurine are ineffective. The comparative effects of these compounds, as well as of the folic antagonist, A-methopterin (4-amino-N^{10} methyl PGA) are shown in Table II. Resistance to 6-mercaptopurine is accompanied by resistance to all other purine and pyrimidine

TABLE II. Effects of Purine Analogs and a Folic Antagonist on Survival Time of Mice Bearing (A) Sensitive Leukemia L 1210 and (B) 6-Mercaptopurine-Resistant Leukemia L 1210. DBA or CDF$_1$ strain of mice.*

Compound	No. separate exp.	Total No. mice	Dosage (mg/kg) Individual	Dosage (mg/kg) Total	Mean survival time, days (range)	% increase in survival
			(A)—L 1210-sensitive†			
Controls	18	145	None		7.9 (7– 9)‡	—
6-Mercaptopurine	15	106	50 –125	250–1000	14.8 (10–22)§	87.3
8-Azaguanine	6	56	75	450– 600	12.4 (10–20)	60.0
8-Azaxanthine	5	48	75 –113	450– 800	8.1 (7–10)	—
2,6-Diaminopurine	3	24	75	450– 600	8.0 (7–10)	—
Thioguanine	4	26	7.5– 10.0	20– 30	13.7 (12–15)	73.5
4-Amino-N^{10} methyl PGA	4	35	3	24	18.1 (14–23)	129.1
"	9	80	3	12	16.7 (13–20)	111.4
			(B)—L 1210-6-mercaptopurine-resistant			
Controls	5	42	None		9.9 (9–11)	—
6-Mercaptopurine	5	42	75	525	10.0 (9–11)	—
8-Azaguanine	3	24	75	600	9.6 (9–11)	—
8-Azaxanthine	4	27	75	600	9.4 (9–13)	—
2,6-Diaminopurine	3	24	75	600	9.8 (9–11)	—
Thioguanine	4	32	7.5	30	9.8 (8–11)	—
4-Amino-N^{10} methyl PGA	2	20	3	24	28.8 (22–38)	190.9
"	2	18	3	12	19.3 (18–22)	95.0

* CDF$_1$ mice = ♀ BALB/c × DBA/2 F$_1$ hybrids. No differences in survival times were noted between DBA and CDF$_1$ mice, nor between sexes.

† Comparable transfer generations were used for these data; for L 1210 sensitive, transfers 202 to 251 and for the transformed, 6-mercaptopurine-resistant line, transfers 10 (210) to 39 (239).

‡ Stand. errors are given in the text.

§ Mean age at death from leukemia of mice receiving total dosage of 6-mercaptopurine at the 250 mg/kg level was 13.2 days; 300-450 mg/kg—15.3 days; 525-600 mg/kg—15.3 days and 750-1000 mg/kg—14.2 days. See text.

antagonists studied. This is unlike the resistance phenomenon in *L. casei* in which resistance to this compound was not accompanied by resistance to other adenine antagonists, particularly 2,6-diaminopurine, 8-Azadenine, and purine and 8-Azaguanine, a guanine antagonist(13).

Dosage levels of 6-mercaptopurine as high as 600 mg/K were without effect on survival time of DBA/2 of CDF$_1$ mice bearing an 8-Azaguanine-resistant line of L 1210, indicating again cross-resistance, whereas the analog provided for near-optimal growth, as determined either by weight of localized lymphomatous tissue or survival time of an 8-Azaguanine dependent line of this leukemia(14).

It has been shown in tests employing *L. casei* that 6-mercaptopurine inhibition of growth is not reversible with folic acid (pteroylglutamic acid)(13). Further evidence that this analog is not an "anti-folic" is shown in the sensitivity of the L 1210-6MR subline to 4-amino-N^{10} methyl PGA. A more striking inhibition of leukemic cell growth by this folic acid antagonist is seen in mice bearing intraperitoneal transplants of the 6-mercaptopurine-resistant subline than in mice bearing the control L 1210-S subline, particularly at near-MTD levels (3 mg/K x 8 days). An increase in survival time of nearly 200% from 9.9 ± 0.10 days to 28.8 ± 0.90 days was obtained whereas an increase from 7.9 ± 0.06 to 18.1 ± 0.69 was shown among controls. It remains to be determined whether this increased sensitivity to a folic antagonist results from a significantly increased requirement for folic acid which is found to be characteristic of a strain of *L. casei* resistant to 6-mercaptopurine(13). It is interesting to note that 2 other transformations in leukemia L 1210, resistant to 8-Azaguanine and dependent upon 8-Azaguanine, are also accompanied by even more striking sensitivity to the folic analog, 4-amino-N^{10} methyl PGA, resulting in increases in survival of leukemic mice over the controls to 400% and more(14). On the other hand, resistant and dependent leukemic cells developed by the use of 4-amino-N^{10} methyl PGA have not shown an increased sensitivity to the purine antagonists.

Extensive attempts to reverse the anti-leukemic activity of 6-mercaptopurine in the L 1210 sensitive line by natural purines have been relatively unsuccessful. Guanine (25 and 50 mg/K x 4) adenine (50, 75, 100, and 150 mg/K x 4), xanthine (100 and 300 mg/K x 4) and hypoxanthine (100, 300, and 500 mg/K x 4) were given intraperitoneally every other day beginning at 24 hours following inoculations of leukemic cells and one hour prior to injections of 50 mg/K of 6-mercaptopurine. In 2 cases significant reversals were seen: 50% reversal, as measured by leukemic death was obtained by adenine at a dosage level of 75 mg/K x 4 and 50% reversal by hypoxanthine (300 mg/K x 4). However, these effects were not consistent from experiment to experiment and are in constrast to results with *L. casei* in which growth inhibition is prevented competitively and apparently with ease by any of these 4 purine bases(13).

No morphologic changes have been observed in the sensitive line of leukemia following exposure to this adenine analog or in the resistant line developed by continuous exposure *in vivo*.

Summary. 1. The adenine analog 6-mercaptopurine has been shown to inhibit leukemic-cell growth of the acute lymphocytic leukemia L 1210 in a definite, regular and reproducible manner. Lower dosage levels of this compound appear to be as effective as higher (near-MTD) levels as measured by the criterion of leukemic death. 2. A resistant subline of leukemia L 1210 has been developed by consecutive passage of leukemic cells in DBA/2 strain mice receiving daily injections of 6-mercaptopurine. Resistant leukemic cells grow optimally either in the presence or absence of the antagonist. 3. Resistance to 6-mercaptopurine is accompanied by resistance to all other purine analogs tested: 8-Azaguanine, 8-Azaxanthine, 2,6-Diaminopurine, and thioguanine, some of which are moderate anti-leukemic agents in the sensitive line of this transplantable leukemia. 4. An increased sensitivity to the folic acid antagonist, 4-amino-N^{10} methyl PGA (A-methopterin) is a characteristic of the resistant variant as well as of 2 other variant lines developed through the use of 8-Azaguanine. 5. Attempts to reverse the anti-leukemic activity of 6-mercaptopurine by the physiologic purines adenine, guanine, xanthine, and hypoxanthine have been relatively unsuccessful.

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14. Unpublished observations.

Received October 15, 1953. P.S.E.B.M., 1953, v84.

Production of Cx-Protein in Rabbits by Cytotoxic Agents. (20664)

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In 1951, Anderson and McCarty confirmed the occurrence in the rabbit of an acute-phase protein analogous to human C-reactive protein(1). They caused this material to be produced in the rabbit in response to the injection of either virulent pneumococci, virulent streptococci or of a vaccine containing killed typhoid organisms. Twelve to 24 hours after the injection of these agents the acute-phase protein could be detected in the sera of the animals.

In the several experiments reported here our aim was to attempt to produce this acute-phase protein (Cx-protein) in rabbits by the use of agents which do not produce an inflammatory response, but which have a direct action on the leukocytes and cells of the reticulo-endothelial system(2). For this purpose X-radiation, nitrogen mustard and amethopterine‡ were used. The rabbit sera containing Cx-protein and a specific antiserum prepared against this substance were drawn into capillary tubes following the method described by Swift, Wilson and Lancefield(3). The quantity of precipitate which resulted from mixture of antigen and antibody was expressed semi-quantitatively as varying from 0 to +++. The specific Cx-protein antiserum was kindly furnished to us by Dr. Harrison Wood of the Rockefeller Institute for Medical Research. The rabbits were bled at the beginning of the experiment and usually daily thereafter. The blood, which was obtained from the ear veins, was allowed to clot overnight in vaseline-lined tubes at room temperature; the serum was separated off the next day. Leukocyte counts were performed daily, but are not recorded here because there was no obvious correlation between the variation in the leukocyte differential counts and Cx-protein production. In these experiments the following results were obtained.

TABLE I. Appearance of C_x-Protein Following X-Radiation.

Rabbit	Hours after treatment 0	24	48	72	96	144	192
R 2357	—	+	—	—	—	—	—
2358	—	+	—	—	—	—	—
2359	—	++	+	+++	+++	+++	Dead

TABLE II. Appearance of C_x-Protein Following Nitrogen Mustard.

Rabbit	Hours after treatment 0	24	48	72	96	120	192
R 2360	—	++	+	—	++	++	++
2361	—	+++	++	—	—	+++	—
2362	—	+	—	—	—	++	—

TABLE III. Appearance of C_x-Protein Following Amethopterine.

Rabbit	Hours after treatment 0	24	48	72	96	120	144	168
R 2360	—	++	—	—	—	—	—	—
2361	—	++	—	—	—	—	—	—
2362	—	++	—	—	—	—	—	—

1. *X-radiation.* Three rabbits were irradiated for 30 minutes with a surface dose of 300 r.§ In 2 rabbits Cx-protein was produced in only small amounts; in the third, which produced more Cx-protein, death occured on the sixth day, and the rabbit was shown to have coccidiosis. (Table I)

2. *Nitrogen mustard.* Three rabbits were injected with nitrogen mustard (Boots); each received a single intravenous injection of 2mg/K. (Table II). It is interesting that in all three rabbits Cx-protein disappeared on the third day and in each case reappeared later.

3. *Amethopterine.* Three rabbits were injected with amethopterine (Lederle); each

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‡ Kindly furnished to us by Lederle Laboratories.

§ The rabbits were kindly irradiated by Prof. Mitchell of the Radiation Laboratory, University of Cambridge.

was given a single injection of 2.5 mg/K. (Table III).

Summary. An acute phase protein (Cx-protein) has been produced in the rabbit by the administration of 3 cytotoxic agents: X-radiation, nitrogen mustard, and amethopterine.

The authors greatfully acknowledge the kind help and advice of Drs. R. R. A. Coombs and R. I. N. Greaves.

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Received October 15, 1953. P.S.E.B.M., 1953, v84.

Effect of Partial Deficiency of Threonine on Enzyme Activity and Fat Deposition in Liver.* (20665)

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The activities of certain liver enzymes are affected by the quality and quantity of the protein and by the amounts of certain amino acids in the diet(1,2). The activity of liver xanthine oxidase, for example, drops when the level of either tryptophan or methionine in the diet is reduced(3,4). Liver fat deposition in animals fed diets *containing choline* is also affected by the quality and quantity of the dietary protein(5,6) and by the levels of individual amino acids. The accumulation of fat found in the livers of rats fed 9% casein diets is reduced when such diets are supplemented with threonine(5,7). Since partial deficiencies of amino acids affect both liver enzyme activity and liver fat deposition a study of the effect of a partial deficiency of threonine on the activities of certain liver enzymes has been undertaken in an effort to demonstrate a correlation between enzyme activity and fat deposition.

The enzyme systems chosen for the initial study were: xanthine oxidase, which is known to be sensitive to alterations in dietary protein; tyrosine oxidase, which is a soluble cytoplasmic enzyme system(8) yielding acetoacetate as an end product of tyrosine oxidation; succinic oxidase, which is a mitochondrial enzyme essential for the operation of the tricarboxylic acid cycle; choline oxidase which is a mitochondrial enzyme not involved in carbohydrate metabolism; and endogenous oxidation and decarboxylation, which give a measure of the oxidative metabolism of endogenous substrates.

Methods. Male weanling rats of the Sprague-Dawley strain weighing from 40 to 50 g were separated into similar groups of 12 animals each and were maintained in individual cages with raised screen bottoms. The animals were fed the experimental diets *ad libitum* and each animal was weighed weekly during the experimental period of 2 weeks. The basal ration was composed of the following: Sucrose, 81.5; casein, 9.0; DL-methionine, 0.3; DL-tryptophan, 0.1; corn oil, 5.0; salts IV(9), 4; and choline chloride, 0.13%. Vitamins were added to provide, in mg per 100 g of ration, thiamine, 0.5; riboflavin, 0.5; niacin, 1.0; calcium pantothenate, 2.0; pyridoxine, 0.25; biotin, 0.01; folic acid, 0.02; vitamin B_{12}, 0.002; and inositol, 10.0. Two drops of halibut liver oil fortified with vitamins E and K(6) were administered orally each week. DL-threonine, when supplemented in the ration, was added at a level of 0.36% and replaced an equal weight of sucrose. After 2 weeks 6 animals from each group were sacrificed for the determination of liver fat. The animals were stunned and decapitated and the livers were excised. Fat was determined by ether extraction of the dried and ground liver (10). The remaining 6 animals were used for the enzyme determinations. The animals were killed as described above. Each liver, immediately after being removed, was chilled in ice and blotted free of moisture. A weighed portion of each liver was homogenized with 5 volumes of ice-cold 0.039 M sodium potassium phosphate buffer (pH 7.3). Xanthine oxidase activity of the homogenates was determined by the method of Axelrod and Elvehjem(11) and choline oxidase by the method of Williams, Litwack and Elvehjem(12). Endogenous oxidation was determined by measuring oxygen uptake of the homogenates in the absence of substrate. Endogenous carbon dioxide evolution was determined by the "direct method" (13). For the determination of succinic oxidase 0.2 ml of a 0.4 M solution of sodium succinate was placed in the side-arm of a Warburg vessel and in the main compartment were placed 0.6 ml of 0.039 M sodium potassium phosphate buffer, pH 7.3, and 1 ml of the homogenate. The total volume was made up to 2 ml by the addition of distilled water to the side-arm. A control vessel containing distilled water instead of succinate was run simultaneously to obtain a correction for endogenous oxidation. For the tyrosine oxidase determination 0.2 ml of a 0.15 M suspension of L-tyrosine and 0.2 ml of 0.15 M sodium α-ketoglutarate were placed in the side-arm, and 1.6 ml of Krebs-Ringer phosphate buffer (pH 7.3) (13), and 1 ml of homogenate were placed in the main compartment. A control vessel containing distilled water in place of L-tyrosine was included. Potassium hydroxide, 0.2 ml of a 10% solution, was placed in the center well of each vessel, except those for the estimation of endogenous carbon dioxide evolution. The *homogenates* were kept in ice after preparation and were pipetted as soon as possible (about 5 minutes after preparation) into chilled, prepared Warburg vessels. The vessels were incubated at 37°C for 10 minutes to reach temperature equilibrium. The substrates or water were added from the sidearms and the stop-cocks were closed. Readings were taken at 10 minute intervals (30 minute intervals for xanthine oxidase) until the activities of the enzymes had reached a maximum and begun to level off. Endogenous respiration measurements (oxygen uptake and carbon dioxide evolution) were contained in all cases for 1 hour. *Xanthine oxidase activity* was calculated according to the method of Axelrod and Elvehjem(11). Succinic oxidase and choline oxidase were calculated from the series of 10-minute readings most nearly approaching a straight line when plotted against time. Tyrosine oxidase, endogenous oxygen uptake and carbon dioxide evolution, which appeared to be particularly unstable systems, usually reached a maximum during the first 10 minute interval and decreased in activity thereafter. Since it was considered that a better index of the activities of these systems would be obtained by taking into account both the actual activity and the stability of the enzyme system, the activity of the tyrosine oxidase system was calculated by averaging the first three 10-minute readings. The activities of the other two systems, endogenous oxygen uptake and carbon dioxide evolution, were calculated by averaging the six 10-minute readings obtained during 1 hour.

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from Swift and Co., Chicago; the Gelatin Research Society of America, Inc., New York City; and the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

The *nitrogen content* of the homogenates was determined by a micro-Kjeldahl method.

The experiment, as outlined above, was carried out once and then duplicated in its entirety. A total of 48 rats was used in these studies.

Results. Results of the growth studies and liver fat determinations are presented in Table I. In both experiments the inclusion of threonine in the basal diet caused an increase in the rate of growth that was accompanied by a substantial decrease in the percentage of fat in the liver. The variation in absolute values from experiment to experiment can probably be accounted for by variations in the nitrogen content of different lots of casein.

Results of the enzyme determinations are presented in Table II. All values have been calculated on the basis of fresh weight of liver since the relative values remain unchanged when they are converted to a liver nitrogen basis (see footnote in Table II). The activities of the cytoplasmic or soluble liver enzy-

TABLE I. Effect of Additional Dietary Threonine on Growth and Liver Fat of Rats Receiving a 9% Casein Ration Supplemented with Methionine and Tryptophan.

	Growth			Liver fat		
Exp. No.	No. of rats	Basal	+ threonine	No. of rats	Basal	+ threonine
		g/wk			%	
I	12	13.7 ± 1.9*	19.5 ± 2.2	6	40.4 ± 2.1	20.7 ± 1.6
II	12	19.7 ± 1.8	25.3 ± 2.2	6	26.9 ± 2.0	12.9 ± 1.1

* Stand. error of mean.

TABLE II. Effect of Additional Dietary Threonine on Liver Enzyme Activity of Rats Receiving a 9% Casein Ration Supplemented with Methionine and Tryptophan.

	Exp. I		Exp. II			Avg of 2 exp.	
Enzyme measured*	Basal	+ threonine	Basal	+ threonine	Total No. of rats	Basal	+ threonine
Xanthine oxidase	10	64	25	50	24	17 ± 6†	57 ± 7†
Tyrosine "	83	307	86	338	20	84 ± 22	322 ± 19
Succinic "	2660	1740	2400	1520	22	2520 ± 200	1620 ± 160
Choline "	1620	1240	1560	960	20	1580 ± 60	1090 ± 122
Endogenous oxidation	1170	2040	855	1695	22	955 ± 190	1870 ± 110
Endogenous decarboxylation	630	1590	473	1155	21	560 ± 90	1370 ± 120

* Values expressed as $\mu l O_2$ or CO_2/g fresh liver/hr. No significant change in relative activity of any of the groups was observed when calculated on the basis of liver nitrogen.

† Stand. error of mean.

mes, xanthine oxidase and tyrosine oxidase, were lower in homogenates from animals receiving the basal diet without additional threonine. The reverse was true, however, of the mitochondrial enzymes, succinic oxidase and choline oxidase. Endogenous oxidation and carbon dioxide production, like the cytoplasmic enzymes, were considerably lower in the livers of animals which received no supplementary threonine.

Discussion. The reduced deposition of fat found in the livers of animals which received the basal diet supplemented with threonine is in agreement with the results of previous reports(5,7). Although the inclusion of threonine in the basal diet caused increased growth, little correlation has been observed between its action in reducing liver fat deposition and its growth-promoting activity(5,6).

Increased activities of the mitochondrial enzymes in the homogenates from animals receiving the basal diet were not anticipated. Since the capacity for oxidation of both succinate and choline is greater and since liver fat deposition is greater when threonine is limiting, these observations do not support the hypothesis that the higher liver fat of animals receiving the basal diet is the result of decreased synthesis of mitochondrial enzymes (7).

The differences observed in xanthine oxidase activity can be correlated directly with the level of threonine in the diet. Since liver xanthine oxidase is known to be sensitive to specific amino acid deficiencies such as tryptophan(3) and methionine(4), and since threonine is the limiting amino acid in the basal diet, these results indicate that the level of dietary threonine is also important in maintaining the activity of this enzyme. The reduced activity of tyrosine oxidase, which is also a soluble enzyme located in the cytoplasm, can probably be similarly explained.

The markedly lower endogenous respiration of liver homogenates from animals receiving the basal diet is indicative of a metabolic difference between the 2 groups. However, since differences in these endogenous measurements may be caused by differences in any one of a number of factors (*e.g.* supply of respiratory substrates, concentration of co-factors, activities of oxidative enzymes) it is not possible to link these changes directly with alterations in liver fat deposition. The possibility that the reduced endogenous respiration may be directly related to the accumulation of liver fat is worthy of further investigation.

It is evident from the experiments reported in this paper that a partial deficiency of threonine causes marked changes in the activities of several liver enzymes as well as in the extent of liver fat deposition. Since these marked changes occur concurrently, it is suggested that the liver fat accumulation observed in animals fed 9% casein-sucrose diets is a reflection of an alteration in the activity of one or more enzymes important for normal liver function.

Summary. The effect of a partial deficiency of threonine in the rat on the activities of several liver enzymes and on liver fat deposition has been determined. Liver fat deposition, in confirmation of earlier reports, was higher in animals receiving the threonine-deficient diet. The activities of the mitochondrial enzymes, succinic oxidase and choline oxidase, were higher in liver homogenates from threonine-deficient animals. Endogenous respiration and the activities of the cytoplasmic enzymes, xanthine oxidase and tyrosine oxidase, were lower in the livers of animals fed diets deficient in threonine.

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Received October 19, 1953. P.S.E.B.M., 1953, v84.

Role of Bile Salts in Activity of Cholesterol Esterase. (20666)

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Nedswedski(1-3) showed that bile salts were required for the synthesis of cholesterol ester from colloidal solutions of cholesterol and higher fatty acids by pancreatic cholesterol esterase. In the absence of bile salts, the enzyme was completely inactivated in 24 hours, but if bile salts were present, it retained its activity almost completely for 10 days. It was postulated that the bile salts exert their effect by stabilizing the enzyme. Klein(4) observed that hydrolytic cholesterol esterase of cattle pancreas was activated by bile acids, especially cholic acid. Nomura(5), Sym(6), and Vercellone(7) reported similar observations. Recent reports(8,9) have presented improved procedures for studying hydrolytic and esterifying cholesterol esterase systems. Both systems were demonstrated in pancreas(8,9) dog serum(10), and rat intestinal mucosa(11). The enzyme or enzymes occurring in these three tissues were alike in respect to optimum pH, inactivation temperature, and in requiring bile salts for activity. Since the substrate emulsions employed in our experiments(8-12) were stable and uniform in composition in absence of bile salts, it seemed unlikely that the effect of bile salts on activity of cholesterol esterase was due to their emulsifying action on the substrates. Also, we observed repeatedly that pancreatic homogenates, stored at 5-7°, retain full activity for 20-30 days in the absence of bile salts. Our experiments have confirmed previously reported requirement of bile salts for cholesterol esterase activity, yet the results have not been in accord with any of the suggested explanations.

In view of this and the possible role of this enzyme in absorption of cholesterol(13) it seemed desirable to investigate further the *mechanism* of the effect of bile salts on cholesterol esterase activity. First, the comparative effect of different bile salts was determined in both the hydrolytic and esterifying cholesterol esterase systems. Of the bile salts tested, sodium taurocholate and cholate were most active. It was observed that bile acids having 3, 2, and 1 hydroxyl groups respectively showed a decreasing order of activity while sodium dehydrocholate with no hydroxyl groups was inactive in promoting either synthesis or hydrolysis. Also evidence was obtained that optimal cholesterol esterase activity occurs when bile salts and cholesterol are present in a ratio of 1:1; an excess of the former produced inhibition of esterifying activity.

Methods and materials. Fresh frozen hog pancreas was used as enzyme source and the homogenates prepared as previously described (11). The substrate mixtures were prepared as described earlier(8,9) and contained cholesterol and oleic acid in experiments on the esterifying cholesterol esterase system and cholesterol oleate in those on the hydrolytic system. The pH of the digests for hydrolysis was 6.5 and for esterification, 6.1. The different bile salts, (amounts indicated in Table I) were added during preparation of substrate mixtures. One cc of pancreas homogenate equivalent to .2 g fresh tissue was added to 12 cc of substrate mixture in all cases. Incubation temperature was 37°C. In experiments on the esterifying system, samples were removed from the enzyme digests (substrate mixture plus pancreas homogenate) at 0 and either 1 or 3 hours; with the hydrolytic system samples were taken at 0 and 4 hours.

TABLE I. Effect of Bile Salts on Cholesterol Esterase Activity.

	Bile salt		Activity*	
Exp.	Type	Conc. (mMol)	Esterification† (mMol)	Hydrolysis‡ (mMol)
1	Taurocholate	.186	.477	.089
	Glycocholate	”	.076	.044
	Cholate	”	.300	.103
	Desoxycholate	”	.031	.043
	Lithocholate	”	.009	.006
	Dehydrocholate	”	.000	.000
2	Taurocholate	”	.493	.079
	Glycocholate	”	.092	.055
	Cholate	”	.293	.093
	Cholate + taurine	”	.293	.095
	Cholate + glycine	”	.281	.095
3	Taurocholate	.000	.000	.000
	”	.065	.204	.040
	”	.086	.301	.045
	”	.129	.362	.062
	”	.258	.407	.068
	”	.516	.255	.078
	”	.774	.169	.095

* Expressed as mMol cholesterol esterified or liberated by hydrolysis/g fresh tissue/hr.

† Substrate: .258 mMol cholesterol and .774 mMol oleic acid/digest in Exp. 1 and 2. Cholesterol, oleic acid, .258 mMol/digest in Exp. 3.

‡ Substrate: .258 mMol cholesterol oleate/digest.

Other pertinent information given in Table I. Throughout the period of incubation the enzyme was saturated with the substrate so that enzyme digests could be compared with one another in the same experiment. Total and free cholesterol fractions were determined by the method of Schoenheimer and Sperry (14) as modified by Sperry and Webb(15). All calculations based on amounts of free cholesterol present in the digests at zero time and at the end of the incubation period. Results are expressed in terms of mmol of cholesterol esterified or liberated by hydrolysis per g of fresh tissue per hour.

Results. Influence of Different Bile Salts on Cholesterol Esterase Activity. Results shown in Exp. 1, Table I. Sodium taurocholate was the most active bile salt in promoting esterification. In the hydrolytic system taurocholate and cholate had approximately the same activity. Glycocholate was markedly less active in promoting either esterification or hydrolysis than taurocholate or the comparable unconjugated salt, cholate. In the series of unconjugated bile salts a decreasing order of activity was observed with cholate, desoxycholate, lithocholate, and dehydrocholate. These bile salts have 3, 2, 1, and 0 hydroxyl groups per molecule, respectively.

Effect of Conjugated Versus Unconjugated Bile Salts. The data on the effect of different bile salts indicated that conjugation of cholic acid with glycine markedly decreased the effect of cholate in both esterification and hydrolysis while conjugation with taurine enhanced its effect in esterification. The purpose of Exp. 2 was to ascertain if the conjugated bile salts function as a unit, or whether the components have independent effects. Sodium cholate exhibited the same degree of activation when alone or when in association with equivalent amounts of either taurine or glycine while the corresponding conjugated bile salts, taurocholate and glycocholate exhibited marked differences in promoting synthesis and hydrolysis in comparison to cholate. It appears that the conjugated bile salts function as a unit and that free taurine and glycine have no effect on the activation of the enzyme by cholate.

Effect of Bile Salt Concentration on Cholesterol Esterase Activity. In previous work (9) it was observed that at a cholesterol level of .195 mmol as the amount of bile salt per digest was increased from 0 to .186 mmol there was a marked increase in the level of activity. Above this concentration and up to .280 mmol there were further increases in activity, but of a smaller magnitude. In the previous study the concentration of bile salts did not extend beyond .327 mmol. In Exp. 3 the cholesterol concentration was held constant at .258 mmol and the taurocholate concentration varied from 0 to .774 mmol. As the bile salt concentration was increased up to .258 mmol, the esterifying and hydrolytic activity increased. Above this concentration of bile salts and up to .774 mmol there was a depression of esterifying activity. In the hydrolytic system further activation occurred with increasing concentrations of sodium taurocholate, up to .774 mmol.

Relationship of Substrate to Bile Salt Concentration. Earlier(9) it was shown that increasing the oleic acid concentration from 1 to 2 molar equivalents of cholesterol, with the bile salt concentration constant, doubled the esterifying activity; further increases in oleic acid concentration did not significantly increase the esterifying activity. Bile salt concentration was optimum for synthetic activity at the level of cholesterol used so it seemed that the oleic acid effect was a mass action effect and was independent of the bile salt concentration. This and the observation reported above that the esterifying activity was maximal with the bile salt concentration at a 1:1 ratio with cholesterol suggested that if the bile salt concentration were held constant and the cholesterol (and oleic acid) varied there should be an increase in activity up to a 1:1 ratio of cholesterol to bile salt; higher levels of cholesterol should have no effect. This type of experiment was run with cholesterol and oleic acid at .065, .129, .258, .516, and .774 mmol per digest with the taurocholate held constant at .258 mmol. The cholesterol esterified, as mmol per g fresh tissue per hour, was .153, .296, .430, .423, and .431, respectively. Thus, the esterification was maximal at a molar ratio of 1:1 for cholesterol and bile salt and there was no effect of higher levels of cholesterol.

Conclusions. The data on the different bile salts suggest that several aspects of the structure of the bile salt molecule are involved in their effects on cholesterol esterase activity. Firstly, taurocholic acid has a distinctly greater effect than either glycocholic or the unconjugated cholic acid (the latter in the esterifying system only). Secondly, conjugation with glycine inhibits the effect of cholic acid. Thirdly, free taurine and glycine have no influence on the effect of cholic acid, *i.e.* the effects of taurocholic and glycocholic acids are due to the intact molecules. Fourthly, in the series of unconjugated acids the degree of effect was greatest with cholic acid which has 3 hydroxyl groups in the molecule and the effect decreased with the decreasing number of hydroxyl groups until with dehydrocholic, which has no hydroxyl groups, there was no effect. The results clearly demonstrate that both the hydroxyl groups and the carboxyl group, free or conjugated, are involved in the effect of bile salts on cholesterol esterase activity.

From the previous work, reviewed above, the bile salt effect does not appear to be related to emulsification of the substrate or the stabilization of the enzyme. The present findings on the interrelationships of cholesterol and bile salt concentration suggest some type of combination between cholesterol and bile salt. The data on the esterification system are especially significant. They indicate that 1 molecule of bile salt is associated with 1 molecule of cholesterol in the reaction with the fatty acid. It seems likely that the levels of activity observed were related to the concentration of a complex between cholesterol and bile salts rather than the concentration of either of the compounds, independently. In the hydrolytic system there was some effect of bile salt concentration above a 1:1 molar ratio with the cholesterol ester. However, the results are in accord with the hypothesis of complex formation between cholesterol or cholesterol ester and bile salt. If the mechanism of the bile salt effect is complex formation, the differences between the different bile salts could be due either to ease of complex formation or a specificity of cholesterol esterase related to the structure of the bile salt molecule.

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Received June 10, 1953. P.S.E.B.M., 1953, v84.

Cortisone Effect on Antibody Levels in Rabbits Simultaneously Immunized with Serum Albumin and Sheep Cells.* (20667)

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We have reported(1) that in a rabbit simultaneously immunized with bovine serum albumin and mumps vaccine, treatment with cortisone might markedly suppress the formation of antibody to the serum albumin while leaving the antibody to mumps apparently unaffected as compared with control animals. The antibody to mumps was measured by 3 different serological tests (hemagglutination inhibition, complement fixation and neutralization) which showed good agreement but all 3 tests are at best semi-quantitative and gave no indication of the actual amounts (weights) of the antibody involved. In order to establish whether cortisone might affect 2 different antigen-antibody systems differently in the same rabbit we have investigated the effect of cortisone on the sheep cell hemolysin system in rabbits, this being a system accessible to quantitative study and one in which preliminary experiments indicated no difference whatsoever in the hemolysin or agglutinin serum dilution titers of animals treated or not treated with cortisone.

Materials and methods. Antigens. Rabbits were immunized simultaneously with alum-precipitated bovine serum albumin and with either whole sheep red blood cells or stromata prepared by laking these cells. In order to give comparable doses of cells and stromata the following procedure was adopted. Approximately one liter of sheep blood was obtained in Alsever's solution and filtered to give a uniform suspension. The volume of packed cells (18%) in this suspension after centrifuging at a definite speed and time (2500 RPM, 10 min.) was noted and aliquots were withdrawn as needed, washed and diluted in saline to give a calculated 5% suspension of cells, the concentration used for immunization. Another aliquot of the cell suspension was then laked, thoroughly washed at high speed until the supernate contained only a trace of N, then suspended in a volume of saline equivalent to that which would have been used to dilute the original suspension aliquot to a 5% concentration of whole cells. *Immunization.* All antigen injections were intravenous and given over a period of 15 days. Eight animals received 7 injections of one mg each of alum-precipitated bovine serum albumin and 7 simultaneous injections of one ml of stromata suspension equivalent to a 5% cell suspension. Of these, 4 were treated with cortisone (Group A) and 4 not (Group B). Nine animals were immunized with BSA as above and with 5% cells as for stroma. Of these, 5 (Group C) were treated with cortisone and 4 were controls (Group D). Cortisone was started 3 days prior to the first antigen injection and continued at a dose of 4 mg daily 5 days per week until the 7th day after the last immunizing injection, at which time the animals were bled by cardiac puncture. During the course of the experiment there were 3 deaths; one each in Groups B, C, and D. *Antibody.* The volumes of sera obtained were estimated and a weight of sodium ethyl mercurithiosalicylate was added to each to give a 1:10000 concentration. Anti-albumin N was determined by the quantitative precipitin procedure(1). Serum precipitin titers were obtained by adding 2 μg of bovine serum albumin to one ml of serum dilution. Quantitative stroma agglutinin N was determined essentially by the method of Heidelberger and Treffers(2) except that the sera were not inactivated at 56°C. Hemag-

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TABLE I. Summary of Antibody Levels in Cortisone Treated and Control Animals.

Group	Weight		mg N anti-BSA*/ml serum	Anti-BSA precipitin tit.	AbN absorbed/ml serum by S.S.†			S.C.‡ agg. tit.	S.C. hemolysin tit.	Agg. for S.C. in supernate, 3rd absorp.	
	At start	At end			1st absorp.	2nd absorp.	Total				
A	2680	2735	<.010	2	.17	0	.17	320	3200	0	
	2630	2680	<.010	4	.16	0	.16	320	1600	+	
	2660	2810	.028	4	.15	.05	.20	320	1600	0	
	2565	2850	.040	8	.12	.01	.13	320	800	0	.17
B	2325	2545	.122	16	.14	0	.14	320	800	+	
	2300	2550	.088	16	.12	.02	.14	320	3200	+	
	2175	2800	.044	8	.14	0	.14	320	1600	+	.14
C	2430	2600	.050	8	.19	0	.19	2560	3200	+	
	2400	2700	.044	8	.20	.02	.22	1280	6400	+	
	2405	2625	.050	8	.24	0	.24	2560	6400	+	
	2350	2650	.068	16	.28	.05	.33	1280	6400	+	.25
D	2350	2550	.122	16	.22	.07	.29	1280	6400	+	
	1975	2360	.236	32	.30	.07	.37	2560	6400	+	
	2500	2975	.184	32	.27	.04	.31	2560	1600	+	.32

Group A—Immunized with BSA and stroma, cortisone-treated. B—Immunized with BSA and stroma, control. C—Immunized with BSA and cells, cortisone-treated. D—Immunized with BSA and cells, control. A third cell absorption is not included in the table since significant amounts of N were not removed from any of the sera. The second absorption was done on 2/3 of the supernate of the first absorption and the values for agglutinin N have been adjusted accordingly.

* Bovine serum albumin (BSA). † Sheep cell stromata (SS). ‡ Sheep cells (S.C.).

glutinin and hemolysin serum dilution titers were done in the usual way using 0.5 ml of serum dilution plus 0.5 ml of 0.25% red cells for the agglutinin titrations and 0.4 ml serum dilution, 0.2 ml of 0.5% cells and 0.2 ml of 1:20 guinea pig complement for hemolysin.

Results. The results obtained are summarized in Table I. All animals were immunized and bled at the same time and each procedure was done on all 14 sera at the same time. The effect of cortisone on anti-albumin N levels is readily apparent and is also reflected in the precipitin titers. The anti-albumin N levels in the cortisone treated animals average only 1/3 of that in the corresponding control group. Groups C and D appear to have higher anti-albumin levels than A and B. While these groups all ran concurrently, they were housed in 2 separate rooms, the room containing Groups C and D being air-conditioned and the other not, while the weather was warm. Whether this accounts for the differences observed or whether the fact that one pair of groups received whole sheep red cells and the other red cell stromata has not been determined.

The agglutinin N presents a different picture. Some uncertainty exists as to the absolute amounts of agglutinin N since, as was found by Heidelberger and Treffers(2), there is a loss of stromata N in the stroma-saline blanks. The values given in Table I are maximum agglutinin N levels obtained by subtracting the N of the stroma-saline blanks from the total N obtained with each serum. The minimum agglutinin N values, obtained by subtracting the total N contained in the aliquot of SS suspension used for absorption[†] would be lower by the same amount for each serum, approximately 0.1 mg N. In any case the values given are strictly comparable.

Comparing the agglutinin N values of groups A and B it can be seen that the cortisone had no effect on antibody produced in response to stroma injection. In fact, the cortisone-treated group A appears to have higher antibody levels than the Group B controls. The agglutinin titers are uniform in both groups and do not reflect the differences in antibody N levels, which is not surprising since these differences are much less than 2-fold. The hemolysin titers on the other hand show a great variation (although no significant difference between the 2 groups) again without reflecting the differences in antibody N among the individual animals of Groups A and B.

A comparison of the agglutinin N values in Groups C and D shows still another picture. There is little difference between the 2 groups insofar as the first absorption is concerned, but the second absorption brings the agglutinin N values for Group D to a slightly higher level than those in Group C. The difference between these groups may or may not be significant—the average of cortisone treated Group C is 0.25 mg N/ml, average of control Group D is 0.32 mg N/ml—but is not at all comparable to the difference in anti-albumin N. The agglutinin and hemolysin titers are not different in the 2 groups. Again these titers do not correspond well with the agglutinin N values in the same animals, but a comparison between the stromata-immunized groups and the cell-immunized groups does show a broad correlation between agglutinin N and titer.

Such a comparison also brings out 2 other points and these are that an average increase of less than 2-fold in agglutinin N values in the cell immunized groups vs. the stroma immunized groups resulted in a 4- to 8-fold increase in agglutinin titer and that the hemolysin titer did not increase to the same extent as the agglutinin titer. The statistical significance of these observations is open to question but they have a reasonable explanation in that the cell immunized animals might be expected to have antibody to antigens not given the stroma immunized animals. This could conceivably lead to a higher agglutinin titer in animals immunized with cells, whereas the agglutinin N value being obtained with stroma would not be affected as much. Speculation along these lines leads to the interesting question of whether more than one sheep cell antigen can give rise to hemolytic antibody and if so, does the hemolysin titer reflect the sum of these antibodies or does it represent only that antibody present in highest concentration.

[†] For the first, second and third absorptions these values are 0.730, 0.366 and 0.382 mg N respectively. Each value being the average of 4 samples taken at intervals during the pipetting of the stromata suspension into the 14 tubes. The maximum deviation from the average was less than ± 2.0% indicating that the stroma suspensions could be pipetted quite accurately.

Discussion. The data indicate that in a rabbit simultaneously immunized with bovine serum albumin N and sheep red cells or stroma, cortisone may suppress antibody to the soluble protein antigen while leaving unaffected the development of antibody to the particulate antigen. Since the effect of cortisone on mumps antibody(1) seems to be intermediate between its striking effect on anti-albumin on the one hand and its complete lack of effect on anti-sheep cell stroma on the other, it is suggested that with increasing particle size of the antigen there is less effect of cortisone on antibody production. This hypothesis is consistent with the fact that while anti-stroma was unaffected there did appear to be a slight effect on antibody to whole sheep cells since an *in vivo* release of soluble antigen from cells could lead to suppression of antibody to this soluble antigen(s) by cortisone.

The fact that Bjørneboe *et al.*(3) have found that antibody to pneumococcal vaccine is suppressed by cortisone would seem to be inconsistent with the particle size idea, but an examination of their data can show otherwise. The route of antigen inoculation used was intravenous, as in this paper, and the rabbits of comparable size. Using a dose of 10 mg of cortisone daily the difference in the average antibody levels of their treated and control groups is less than the difference found above and previously(1) in the anti-albumin levels of animals not treated or receiving 4 mg of cortisone daily. With a dose of cortisone more nearly comparable to that used here, 2.5 mg daily, Bjørneboe *et al.* found much less suppression of antibody than is characteristic for anti-albumin and considerable overlapping of individual treated and control levels, a situation similar to that described above for antibody to whole sheep cells. It is entirely possible that whatever suppressive effect cortisone exerts on antibody to pneumococcal vaccine is directed against antibody to soluble constituents (*in vivo*) of the pneumococcus. Moreover, instances may be found in the literature where immunization with bacterial products is affected by cortisone or ACTH, whereas immunization with whole bacteria is not (*e.g.*, 4, pp. 20, 52, and 87).

The most basic objection to the antigen size idea comes from the fact that albumin, mumps virus and red cells differ not only in size, but also of course chemically and that while bovine serum albumin may be regarded as a single antigen the red cell and possibly the mumps virus are in reality multiple antigens. What is needed is a single antigen in very different particle sizes. An approach to this might be made by conjugating bovine serum albumin and red cells with some chemical grouping (*e.g.*, phenylisocynate) which destroys the original specificity of the antigen and results in antibody directed primarily against the conjugated group(5). This study is in progress.

Summary. In rabbits simultaneously immunized with bovine serum albumin and sheep red cells or stroma, cortisone suppressed the formation of antibody to bovine serum albumin but had little or no effect on the production of hemolysin or agglutinin as measured by serum dilution titers and quantitative agglutinin N.

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Received August 24, 1953. P.S.E.B.M., 1953, v84.

Studies in Procollagen. I. Solubility.* (20668)

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In the speculations regarding the mechanism of formation of collagen fibers, the existence of a soluble collagen precursor has frequently been suggested. Such a possibility was amplified by the demonstration by Nageotte(1) that collagen fibrils could be precipitated by sodium chloride from dilute acetic acid solutions obtained from rat tail tendon. More recently Orekhovitch *et al.*(2) have demonstrated that treatment of mammalian skin with citrate buffers (pH 3.0-4.0) extracts a protein which readily forms fibrils resembling collagen both morphologically and in amino acid composition (3-6). These workers have named this substance "procollagen" because it appears to be a metabolic precursor of collagen.§ Although interactions of collagen with hyaluronic acid (HA), chondroitinsulfuric acid (CSA), and with heparin have been observed, it has not yet been demonstrated that these acid mucopolysaccharides are combined with collagen in ground substance or participate in fibrogenesis (4,5,7-9) The possible existence of a soluble collagen precursor in connective tissue is of great importance with respect to the metabolism of collagen as well as the behavior of ground substance. In view of the demonstrated existence of the above named polysaccharides their possible interaction with soluble collagen precursors is obvious. Most investigations have been concerned with various means of producing collagen fibrils from solutions of procollagen. It seemed of importance, however, to determine the factors which may be responsible for increasing the solubility of this protein and its possible interactions with other components of ground substance.

This report presents experiments concerned mainly with the effect of certain small ions on solubility of procollagen.

Experimental. Preparation of procollagen. After sacrifice by air embolism, adult albino rabbits were immediately skinned. After removal of the subcutaneous layer, the skin was ground in an electrical meat grinder and either stored in the deep freeze (for periods up to 2 months) or immediately extracted by the procedure of Orekhovitch, *et al.*(2). This involved extraction by stirring at 1° with a 5-fold volume of 0.3 M disodium phosphate for 24 hours. The supernatants obtained after centrifugation were combined and dialyzed against distilled water at 1°. The "globulins" were precipitated by 50% saturation with ammonium sulfate and removed by centrifugation; the "albumins" were then precipitated by saturation of the supernatant with ammonium sulfate. The residue was washed with small amounts of citrate buffer at pH 4.0 and then extracted 6 times with 5-fold portions of McIlvaine (phosphate-citrate) buffer of pH 4.0 by stirring at 1° for 24 hours.‖ Each

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* This investigation was supported by grants from the Helen Hay Whitney Foundation, Chicago Heart Association, and Variety Club of Ill.

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‡ During tenure of a Research Fellowship of American Heart Assn.

§ Metabolic studies of procollagen of the rat have been carried out with the use of carboxyl-labeled glycine by Orekhovitch, *et al.*(2). They concluded therefrom that animal skin contains a series of procollagens which are metabolic precursors of collagen. The same conclusion was reached by Harkness and Neuberger(6) and was based upon similar metabolic experiments with the rabbit.

‖ Electron micrographs of the skin residues remaining after each extraction showed that extraction with disodium phosphate solutions caused little, if any, change in the structure of the collagen fibril. However, extraction with citrate buffer had a progressive effect resulting in very extensive swelling and disorganization of the collagen fibrils. A similar disintegration of collagen fibrils is obtained by treatment of rat tails tendon with dilute acetic acid(10). It may be that procollagen is a structural component of the collagen fibril. We are grateful to Dr. Friedrick Wasserman of the Argonne National Laboratories for aid with electron microscope studies.

extract was dialyzed against a large excess of 0.01 M disodium phosphate at 1°. The voluminous, fibrous precipitate was washed several times with distilled water and stored moist at 4°. Solutions of the above preparations were obtained by extraction with citrate buffers at pH 3.0 or 4.0 followed by centrifugation for 1 hour at about 20,000 times gravity. The protein content was determined by nitrogen analysis and calculated, based upon a nitrogen content of 16%. Even after prolonged centrifugation at 20,000 times gravity the solutions were frequently faintly hazy. However, it was observed that procollagen which yielded clear solutions could be obtained if dialysis of the extract was allowed to proceed against the same buffer in 2 stages, the first stage yielding a small precipitate of material which was discarded, the second yielding the bulk of the precipitable protein.

Uniformity of procollagen preparations. Procollagen, unlike most animal proteins, yields highly viscous solutions at low concentrations. It appeared that viscosity measurements would furnish an index to uniformity and relative freedom from contamination. Procollagen preparations obtained in successive extracts from skin were dissolved in McIlvaine buffer pH 4.0 and adjusted to a concentration of 0.060%. The viscosity relative to buffer (ratio of respective flow times) was determined for each solution in an Ostwald viscometer at 30°. It was found that the viscosity of a solution declined with time at 30° but appeared to become constant after about one hour. For the 1st, 2nd, 5th and 6th extracts the relative viscosities obtained were respectively 2.00, 2.19, 2.15 and 2.24 indicating that, with the exception of the first extract, the preparations were of uniform viscosity (within experimental error). The ultraviolet absorption spectrum of preparations from six successive extractions of skin all closely resembled those reported for gelatin(11), and were similar to those previously reported for procollagen preparations(2). In no case was there any indication of a tyrosine peak in the range of 278-282 mμ.

Solubility of procollagen. Procollagen is soluble in dilute acid, insoluble at neutral or alkaline pH and readily precipitated by low concentrations of neutral salts(2,12). The effect of pH and ion concentration on the solubility of procollagen was further investigated by two separate technics. In a dilution technic, a concentrated solution of procollagen in citrate buffer was mixed with solutions of various ions, whereas in another more direct method, precipitated collagen was shaken with the solution to be tested. In either case the solutions were centrifuged and the amount of dissolved protein calculated from the nitrogen content. A 0.7% solution of procollagen (2nd extract) in McIlvaine buffer, pH 4.0, was dialyzed in a cellophane casing against 100 volumes of 0.1 M citrate or acetate buffer at 4° for 24 hours. Precipitation of protein was noted only in the solution dialyzed against the pH 5.42 acetate buffer. The contents of each casing were diluted 10-fold with distilled water and allowed to stand at room temperature for 3 hours. Precipitates were removed by centrifugation and supernatants were analyzed for nitrogen. Table I shows the amounts of procollagen remaining in the supernatants in the 0.01 M buffers at several pH's. The data suggest that at 0.01 M buffer concentration procollagen is equally soluble in the presence of citrate and acetate ions. However, at higher buffer concentration citrate is more effective than is acetate at pH's above 5.4. A separate dialysis experiment with 0.1 M acetate buffer at pH 5.4 showed that less than 0.15% procollagen was in solution as compared to 0.7% in 0.1 M citrate buffers of pH 6.0 and 7.1. In other experiments, a 0.5% solution of procollagen in 0.1 M citrate buffer pH 6.8, was diluted 10-fold with various solutions to yield a final pH of 5.8 to 6.2. All of the protein remained in solution when the diluent was 0.1 M citrate buffer or 0.1 M $CaCl_2$. Less than 10% of the protein remained dissolved when the diluting solution was 0.1 M $NaHCO_3$,

TABLE I. Effect of pH on Solubility of Procollagen in 0.01 M Citrate and 0.01 M Acetate Buffers.

Citrate		Acetate	
pH	Protein in sol., %	pH	Protein in sol., %
4.24	.065	3.92	.057
4.98	.003	4.86	.030
6.02	.002	5.42	.002
7.08	.002		

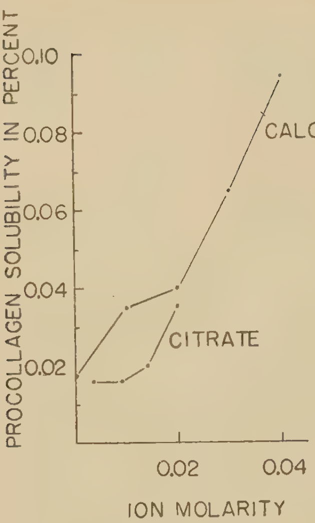

FIG. 1. Effects of citrate and calcium concentration on solubility of procollagen in Krebs-Ringer-carbonate, pH 7.4-7.5 by the dilution method. The solutions containing various amounts of added calcium also contain 0.003 M citrate.

0.1 M NaCl solutions containing 0.3% skin albumin, 0.3% skin globulin, or 0.1% mixed acid mucopolysaccharides from rabbit skin.

Effect of calcium and citrate on solubility. Procollagen in 0.03 M citrate, pH 6.8, was diluted with a solution of Krebs-Ringer-carbonate buffer of pH 7.4-7.5 containing various amounts of citrate and calcium. From the results shown in Fig. 1, it appears that calcium and citrate exert a strong solubilizing effect on procollagen. It was also found that phosphate inhibited the solubilizing action of calcium on procollagen. This action of phosphate may be interpreted as due to association with calcium in competition with procollagen. The increased solubility of procollagen in the presence of calcium can be reversed by dialysis of the solution against the same buffer without calcium. Preliminary experiments had indicated that the rate of solution of protein from the first extract was slightly greater than that of material from succeeding extracts. This finding suggested the possible presence of a small amount of contaminating protein in procollagen from the first extract. When different amounts of protein (pooled fractions) were shaken for 8 hours at 0° with a fixed volume (5 ml) of Krebs-Ringer-carbonate buffer of pH 8.5 containing 0.02 M citrate but no calcium, the amount of protein in the supernatant increased with increase in the amount of solid protein taken in the test (Fig. 2). No difference was found between solubility values obtained in 8 hours or in 16 hours. It appears possible that the contaminating protein is a minor component (estimated as about 3%) with a solubility greater than that of procollagen. Thus, in the following solubility experiments it would be expected that a small but fixed amount of impurity would go into solution, but that changes in protein content of the supernatant found would be due only to the changed solubility of procollagen. Moist procollagen (combined extracts) was shaken with Krebs-Ringer-carbonate solutions at 0° for 8-24 hours. Between 60 and 70 mg of total protein was taken for each 5 ml of solution. After 8 hours, the samples were centrifuged at 0°, and a small amount of supernatant removed for analysis. The remainder of the material was shaken for an additional 8 or 16 hours and the supernatants analyzed once more. No change in solubility was noted after 8 hours.

The results are shown in Fig. 3 and 4. In the presence of citrate, calcium addition at first reduces the amount of procollagen dissolved, but when present in molar excess over citrate, increases the protein solubility. This mutual antagonism of calcium and citrate suggests that the free ionic concentrations of these

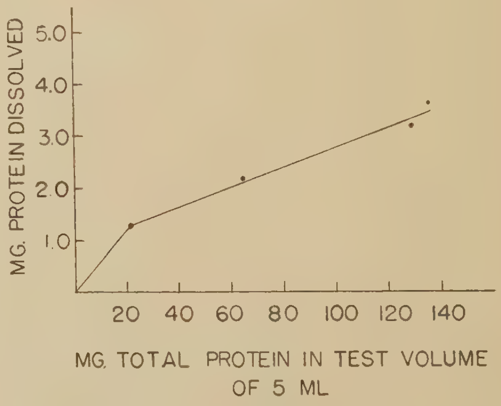

FIG. 2. Effect of total protein present on solubility. Protein present in supernatant after 16 hr at 0° when various amounts of procollagen pooled from first 3 extracts are shaken with 5 ml of Krebs-Ringer-carbonate solution, pH 8.5, containing 0.02 M citrate and no calcium.

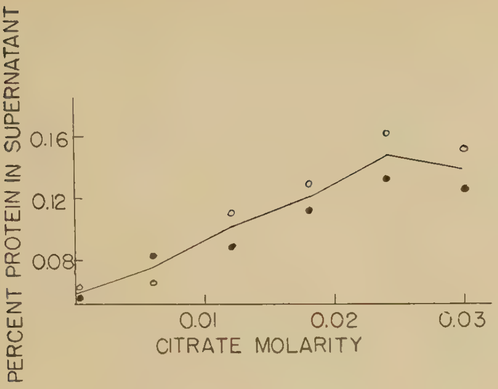

FIG. 3. Solubility of procollagen in Krebs-Ringer-carbonate solution with varying citrate concentrations. No calcium was present and pH was 7.8 to 8.0. Solubility after 8 hr indicated by ○; after 16 hr by ●.

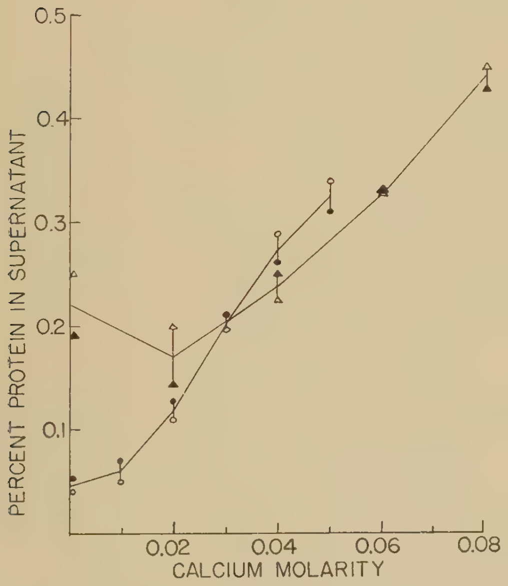

FIG. 4. Solubility of procollagen in Krebs-Ringer-carbonate solution with varying calcium concentrations. The pH was 6.7 to 6.8. Solubility in absence of citrate after 8 hr indicated by ○; after 24 hr by ●. Solubility in presence of 0.03 M citrate after 8 hr indicated by △; after 16 hr by ▲.

substances have been reduced by complex formation with each other.

It is of interest that glucuronic acid had a definite but much weaker solubilizing action on procollagen than does citrate. Under comparable conditions, 0.1 M glucuronic acid is equivalent in effect to 0.012 M citrate (Fig. 3). In these experiments, however, an unknown amount of the uronic acid is present as lactone. Chondroitinsulfuric acid from bovine cartilage in concentrations up to 1% or mixed acid mucopolysaccharides from rabbit skin (0.3%) had little or no effect on the solubility of procollagen in neutral Ringer-carbonate buffers.

Discussion. The solubilizing action of citrate may be interpreted as due to binding of citrate by procollagen, which reduces the electrostatic attractive forces between procollagen molecules in the fibril. A similar interpretation may be given for calcium ion. However, an alternative explanation may also be offered based upon the assumption that part of the strong molecular interaction in procollagen is due to the presence of an acid mucopolysaccharide which interacts with calcium(13). Some evidence in support of this assumption is given by the experiments of Jackson(8) who showed that prior treatment of collagen (calf tendon) with hyaluronidase (presumably to remove chondroitinsulfuric acid) very greatly increased the amount of collagen brought into solution by dilute acetic acid. Thus, the absence of demonstrable effect of added acid polysaccharides on the solubility of procollagen should not be taken to exclude the possibility of interaction between procollagen and acid mucopolysaccharides already present in the protein preparation.

Summary. 1. Procollagen has been prepared from rabbit skin and its solubility characteristics studied. 2. Both citrate and calcium ions exert a strong solubilizing action on procollagen. Added acid mucopolysaccharides from skin and chondroitinsulfuric acid from cartilage show little, if any, influence on the solubility of procollagen.

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Received August 26, 1953. P.S.E.B.M., 1953, v84.

Influence of Dietary Bile Salts on Blood Cholesterol Levels. (20669)

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Mueller(1) carried out the first critical experiments on the role of bile in absorption of cholesterol. When a cholesterol or cholesterol oleate emulsion was fed to dogs in which the bile was diverted from the intestinal tract there was no significant rise in the total cholesterol of the lymph as compared to normal dogs. Schoenheimer(2) also observed that the serum cholesterol of rabbits was increased more after feeding cholesterol with desoxycholic acid than after cholesterol alone. Kim and Ivy(3) found that the serum cholesterol of rats was significantly raised when cholic acid was added to a diet containing cholesterol and oleic acid. When bile acid was added to a cholesterol-free diet no significant increase in blood cholesterol was observed. Recently Siperstein *et al.*,(4) reported that when labeled cholesterol was fed to bile fistula rats none of the labeled compound could be recovered in the lymph. It was suggested that bile was obligatory for the absorption of cholesterol. In addition to bile, pancreatic secretions and fat have been shown to be intimately related to cholesterol absorption(5,6). There is good evidence that esterification of cholesterol is an important step in its absorption and that this process takes place in the intestinal wall(7,8) under the influence of pancreatic cholesterol esterase. In connection with studies (9-11) on this enzyme, it has been demonstrated that it will synthesize and hydrolyze the long-chain fatty acid esters of cholesterol only in the presence of bile salts. In the preceding paper(11) the effect of different bile salts on cholesterol esterase activity *in vitro* was demonstrated. The type and amount of bile salt present in substrate mixture is important in the activity of the enzyme. Bile salts having 3, 2, and 1 hydroxyl groups respectively showed a decreasing order of activity while dehydrocholate with no hydroxyl groups was found to be inactive in promoting either synthesis or hydrolysis. Also the conjugated bile salts, taurocholate and glycocholate showed differences in activity when compared to cholate.

The purpose of the present investigation was to determine, using the blood cholesterol level as an indicator of cholesterol absorption, if different bile salts had different effects on cholesterol absorption comparable to the relations between bile salt structure and cholesterol esterase activity *in vitro* demonstrated in the preceding paper(11). If comparable effects could be demonstrated this would be added evidence that the requirement of bile salts for the absorption of cholesterol is due to its being necessary for cholesterol esterase activity and that cholesterol esterase is directly involved in cholesterol absorption.

Methods and materials. Forty-five rats from our stock colony and raised on Purina pellet chow were used. The animals were housed in separate cages and fed the experimental diets and water *ad libitum*. Following a control period on pellet chow during which the blood cholesterol fractions were determined, the rats were divided into two series.

TABLE I. Feeding Regimen and Dietary Intake of Cholesterol, Bile Salts, and Olive Oil.

Series	No. rats	Group	Days on diet	Bile salt	Dietary intake/day/rat* Cholesterol, mg	 Bile salt, mg	 Olive oil, cc
I	5	E	21	0	200–250	0	2.5–3.
	5	F	21	Cholate	”	100–150	”
	5	G	21	Taurocholate	”	”	”
	5	H	21	Desoxycholate	”	”	”
	5	I	21	Dehydrocholate	”	”	”
II	10	K	7	Dehydrocholate	150–200	75–100	2.0–2.5
	8	K	7	Taurocholate	”	”	”
	8	K	5	Dehydrocholate	”	”	”
	10	L	21	0	”	0	”

* Range of wt of rats in Series I was 225-300 g and in Series II, 125-175 g.

In Series I four groups were fed a basal diet containing 2% cholesterol plus 1% of different bile salts. One group served as a control and received the basal diet with no bile salts. In Series II, two groups (K and L) were fed a basal diet containing 2% cholesterol, with group K receiving in addition 1% of bile salts in the diet successively for 7, 7, and 5 day periods. The basal diet had the following composition: 20% casein, 23% starch, 23% glucose, 25% olive oil, 5% Hubbel-Mendel-Wakeman salt mixture, 2% ruffex, 2% cholesterol, and adequate amounts of the crystalline vitamins. The bile salts were added to the basal diet at the expense of the starch and glucose. Table I shows the feeding regimen and the approximate dietary intake of cholesterol, bile salts and olive oil per rat in Series I and II. The rats were bled from the tail. The blood samples were collected directly into .2 cc pipettes and placed in 1:1 acetone alcohol for cholesterol analysis. Free and total cholesterol were determined on the whole blood by the method of Schoenheimer-Sperry(12) with modifications(13).

Results. Series I. The results are shown in Fig. 1. The total blood cholesterol increased significantly in all groups after one week on the diets. This increase was predominantly in the ester fraction. In comparing the effect of the different bile salts on the blood cholesterol during this initial period, it can be seen that the blood chcolesterol in the group receiving sodium dehydrocholate did not rise significantly above that of the control group not receiving bile salts. The groups receiving cholate, taurocholate, and desoxycholate showed increases in total blood cholesterol of 41.1, 36.9 and 16.5% respectively when compared to the group not receiving bile salts in their diet. During the remainder of the feeding period, the total, the ester and to a lesser extent the free cholesterol fractions continued to rise in the blood of the cholate, taurocholate, and desoxycholate groups. The blood cholesterol of the dehydrocholate and control groups remained about the same. At the end of the feeding period (21 days) the total blood cholesterol levels in the bile salt groups ranged from 280 to 320 mg% and the ester fraction from 170 to 210 mg%. The dehydrocholate and control groups had total cholesterol levels of 145 and 137 mg% and ester levels of 58.6 and 67.6 mg% respectively. It is also interesting to note that the ratio of ester to total cholesterol rose from an average pre-diet value of 26 to 63% in the cholate, taurocholate, and desoxycholate groups. During the same period, the dehydrocholate and control groups showed an increase from 26 to 47% in that ratio. After 21 days the animals were taken off the experimental diets and returned to pellet chow. Within 5 days the blood cholesterol in all groups dropped very markedly, approaching the pre-diet level.

Series II. The effects of different bile salts demonstrated in Series I, especially after 7 days, exhibited a pattern in general agreement with the results obtained in the study on the *in vitro* effect of bile salts in cholesterol esterase activity. It was especially significant that dehydrocholate did not have any effects on the blood cholesterol. In Series I the bile salts were fed to different animals.

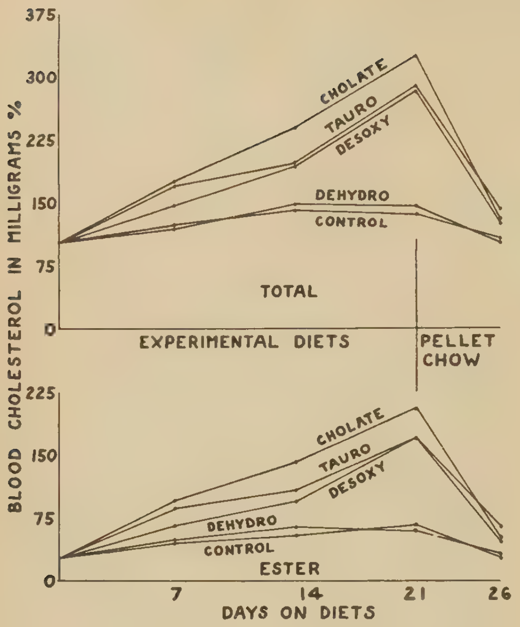

FIG. 1. Total and ester blood cholesterol levels of rats fed different bile salts. Each point represents avg of 5 animals.

It was of further interest to determine if similar differences between different bile salts could be demonstrated in the same animal. The experiment was also carried out to determine if the animals would respond to changes in the bile salt composition of the diet thereby providing evidence that the changes in the blood cholesterol observed in Series I were readily reversible and not due to a pathologic effect of the bile salts. The results are shown in Fig. 2. After 7 days both control and bile salt groups showed significant increases in total and ester blood cholesterol. However, both groups showed the same degree of elevation. This confirms the findings in the previous experiment, namely, that dehydrocholate is ineffective in raising the blood cholesterol level above that of the control group. The bile salt group was then switched to a diet containing sodium taurocholate. After 7 days on this diet, the total blood cholesterol level increased markedly from 131 to 300 mg%. During the same period, the total blood cholesterol of the control group increased from 127 to 160 mg%. It is also interesting to note that the blood cholesterol increased much more rapidly than in the previous group fed sodium taurocholate. Since the animals were younger in the present group, age may be an important factor.

In the final feeding period the bile salt group was returned from the diet containing sodium taurocholate to the diet containing sodium dehydrocholate. After 5 days on that diet both the total and ester blood cholesterol decreased sharply. The total cholesterol decreased from 300 to 215 mg%. The cholesterol fractions in the control group remained at about the same levels. The decline in bood cholesterol observed when the bile salt group was returned to the dehydrocholate diet demonstrates that the effect of bile salts is reversible and that it is unlikely that permanent damage to the animals had taken place. It is also of interest to mention that the feeding periods were of such a short duration that toxic effects of the bile salts were not indicated by examination of the livers. No difference in weight or gross appearance of the livers of the bile salt and control groups was noted in the animals in Series I and II.

Discussion. Previous work relating to the influence of bile on the absorption of cholesterol has been carried out on animals in

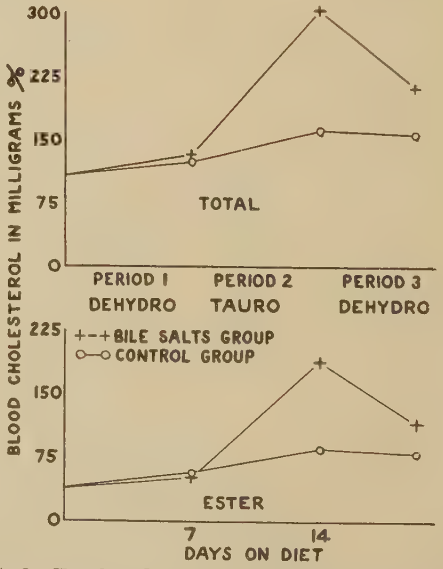

FIG. 2. Total and ester blood cholesterol levels of rats fed sodium dehydrocholate, taurocholate, and dehydrocholate successively. Each point represents avg of 8-10 animals.

which bile has been diverted from the intestinal tract(1,4). However, in studying this process, 4 factors must be considered, namely, pancreatic cholesterol esterase, cholesterol, fat, and bile. It seems likely that each of these components must be present and in certain optimum proportions to each other for maximum absorption of cholesterol. Thus, if there is an excessive amount of cholesterol ingested, sufficient fat and bile must be present for complete absorption. In this series of experiments we have used the intact animal and supplied high levels of cholesterol and fat in the diet. There was a slight increase in the blood cholesterol level during the experimental periods in the animals on the control diet which, according to the above, could be due to the effect of the rats own bile salt supply on the absorption of the dietary cholesterol. In the animals receiving the bile salt diets the markedly higher blood cholesterol levels were due to the effect of the dietary bile salts on the absorption of the dietary cholesterol. This view, which is in accord with the available data, leads to the interesting possibility that the level of the blood cholesterol is controlled, in part, by the amount of bile salts excreted into the intestine.

The present findings indicate that the structure of a bile salt has an important relationship to its effect on the blood cholesterol. It is especially striking that the feeding of dehydrocholate had no effect on the blood cholesterol. This correlates with the finding in the previous paper(11) that dehydrocholic acid, which has no hydroxyl groups, has no effect on cholesterol esterase activity, *in vitro*. It provides further evidence that esterification of cholesterol is required for its absorption and that bile salts are essential for that esterification.

Summary. Different bile salts were fed to rats on high cholesterol-fat diets. The blood cholesterol increased very markedly with the feeding of sodium cholate and taurocholate, somewhat less with the feeding of desoxycholate and no increase was observed in those fed dehydrocholate. When sodium dehydrocholate, taurocholate, and dehydrocholate were fed successively to the same animal, taurocholate increased the blood cholesterol very markedly while dehydrocholate first had no effect and later lowered it. The results of this study agree in general with the findings of the preceding study on the *in vitro* effect of bile salts on cholesterol esterase activity, in that a comparable pattern of activity was observed in relation to the effect of bile salts on the blood cholesterol. It is suggested that the bile salts are necessary for cholesterol absorption because they are a necessary co-factor for cholesterol esterase activity.

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Received September 14, 1953. P.S.E.B.M., 1953, v84.

Effect of Trypsin on Prothrombin.* (20670)

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It has long been known that trypsin clots plasma(1,2) and that it speeds the clotting of whole blood(3). Furthermore, it has been demonstrated that the rapid intravenous injection of trypsin causes intravascular clotting(4,5). The mechanism of the clotting effect of trypsin has not been completely clarified. A number of reports state that trypsin acts on prothrombin, converting it into thrombin(4,6,7), but there is disagreement as to the need for calcium in this reaction. Eagle and Harris(4) believed that calcium is not essential for conversion of prothrombin by trypsin, while Ferguson and associates(6,7) have stressed the essentiality of calcium. A more complex view of the clotting action of trypsin was presented by Lenggenhager(8). He thought that trypsin activated a hypothetical plasma factor and that this factor, together with calcium, converted prothrombin into thrombin. More recently, Schultze and Schwick(9) demonstrated that trypsin speeds the activation of purified prothrombin in concentrated citrate solutions. This effect was also noted for prothrombin solutions in distilled water, 50% glycerin, and 20% serum albumin. However, the role of calcium in the reaction was not considered.

Experiments have been performed which were designed to clarify the mechanism of the clotting effect of trypsin. Three new approaches to the problem were used. The first was the use of purified clotting factors; the second was the use of soybean trypsin inhibitor(10) to neutralize tryptic activity at any desired time; and the third was the use of a quantitative prothrombin assay.

Methods. Prothrombin assays. These were performed by the 2-stage method of Warner, Brinkhous, and Smith(11), as modified by Flynn and Coon. Their modifications involve the use of the purified thromboplastic "accessory factors" needed for the conversion of prothrombin into thrombin(12). *Thrombin assays.* Thrombin solutions were assayed by the second stage of the 2-stage prothrombin assay. *Activation of prothrombin by trypsin.* Trypsin[†] was added to purified prothrombin[‡] in various reaction mixtures. The exact composition of these mixtures is described under Fig. 1. At measured intervals, aliquots of the mixtures were removed and immediately added to trypsin inhibitor.[§] The use of inhibitor prevented the trypsin from acting during the subsequent procedures. Thrombin and prothrombin assays were then performed. Calcium and the thromboplastic "accessory factors" (labile factor (factor V, etc.) and stable factor (factor VII, etc.) (13,14))[‡] were added to the prothrombin along with the trypsin to study their effect on the activation reaction. *Plasma prothrombin utilization.* This was determined by assaying plasma prothrombin concentration before and after addition of trypsin to the plasma. *Antithrombic activity of heparin.* Purified thrombin (Parke, Davis & Co.) was added to oxalated plasma containing heparin (sodium salt, Eli Lilly & Co.). The same procedure was performed with control oxalated plasma without heparin. At measured intervals, an aliquot was removed from each mixture and a thrombin assay done. The reduction in thrombin concentration of the heparinized plasma be-

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* Supported by a grant-in-aid from the Armour Laboratories, Chicago, Ill.

The laboratory facilities of the Department of Pathology, College of Physicians and Surgeons, were generously made available, including the constant advice of Drs. Joseph E. Flynn and Robert W. Coon of that Department.

[†] A twice-recrystallized, salt-free, and lyophilized preparation was used (Worthington Biochemical, Freehold, N. J.).

[‡] Prothrombin, labile factor, and stable factor were kindly supplied by Drs. J. E. Flynn and R. W. Coon. Methods of preparation are described elsewhere(12).

[§] A 5-times recrystallized preparation of soybean origin was used (Worthington Bio.).

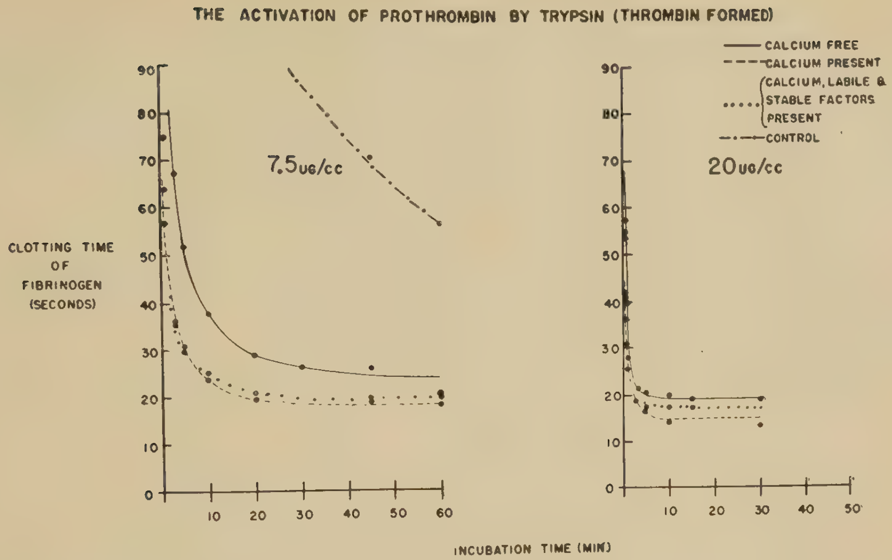

FIG. 1. Activation of prothrombin by trypsin (thrombin formed). *Reaction mixtures:* (a) *calcium-free:* 0.5 ml prothrombin sol. (diluted to 340 units/ml with 7.5% acacia in oxalated 0.85% saline) + 1.4 ml 7.5% acacia in oxal. 0.85% sal. + 0.1 ml trypsin in oxal. 0.85% sal.; (b) *calcium added:* 0.5 ml proth. sol. (dil. to 340 units/ml with 7.5% acacia in 0.85% sal.) + 1.4 ml 7.5% acacia in 0.25% $CaCl_2$ and 0.85% sal. + 0.1 ml trypsin; (c) *calcium, labile and stable factors added:* 0.5 ml proth. (prep. as in b) + 1.2 ml 7.5% acacia in 0.4% $CaCl_2$ and 0.85% sal. + 0.1 ml labile factor + 0.1 ml stable factor + 0.1 ml trypsin; (d) *control-calcium, labile, and stable factors added, but no trypsin:* same as in *c* except that 0.1 ml of 0.85% sal. without trypsin was used. 0.2 ml aliquots were removed and added to 0.2 ml of 0.85% sal. buffered to pH 7.4 and containing trypsin inhibitor in an amt equal to the amt of trypsin added to the react. mixture.

low that of the unheparinized plasma was considered to be an estimate of the increase in antithrombic activity of the heparinized plasma.

Results. The experiment illustrated by Fig. 1 demonstrates that trypsin, by itself, can cause the conversion of prothrombin into thrombin. Within the range of trypsin concentrations used, the rate of this reaction varies with the concentration of trypsin. In addition, calcium is shown to increase the rate of thrombin formation. However, the total amount of thrombin formed is unaffected by the presence of calcium. Furthermore, the thromboplastic "accessory factors," together with calcium, have no effect other than that produced by calcium alone. Although the data presented in Fig. 1 represent trypsin concentrations of 7.5 and 20 μg/ml, essentially similar results were obtained with concentrations of 15 and 50 μg/ml. However, with 50 μg/ml, prothrombin and thrombin activities in the reaction mixtures were rapidly lost. The control mixture, represented by the dash-dot line curve, consisted of purified prothrombin, calcium, labile factor and stable factor, but no trypsin. It will be noted that some thrombin formed in this mixture but the amount was small. This was undoubtedly due to thromboplastic impurities in the reagents. When trypsin was added to such a mixture (dotted line curve), thrombin was formed in considerably greater amounts.

In the above experiment on the activation of prothrombin by trypsin there was a discrepancy between the amount of prothrombin present and the amount of thrombin formed. The available prothrombin could, if fully converted, have given rise to a greater amount of thrombin than that recovered. The question arose as to whether some of the prothrombin had been digested or had remained unconverted. To answer this question, residual prothrombin concentrations were determined at various stages of the reaction. Fig. 2 illustrates the results graphically. As

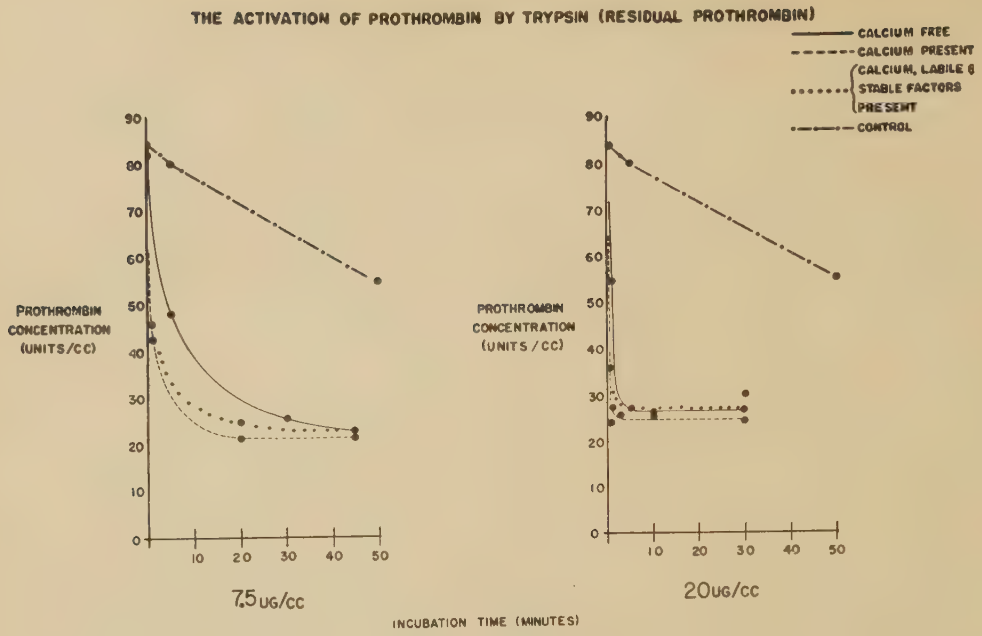

FIG. 2. Activation of prothrombin by trypsin (residual prothrombin). Reaction mixtures same as for Fig. 1.

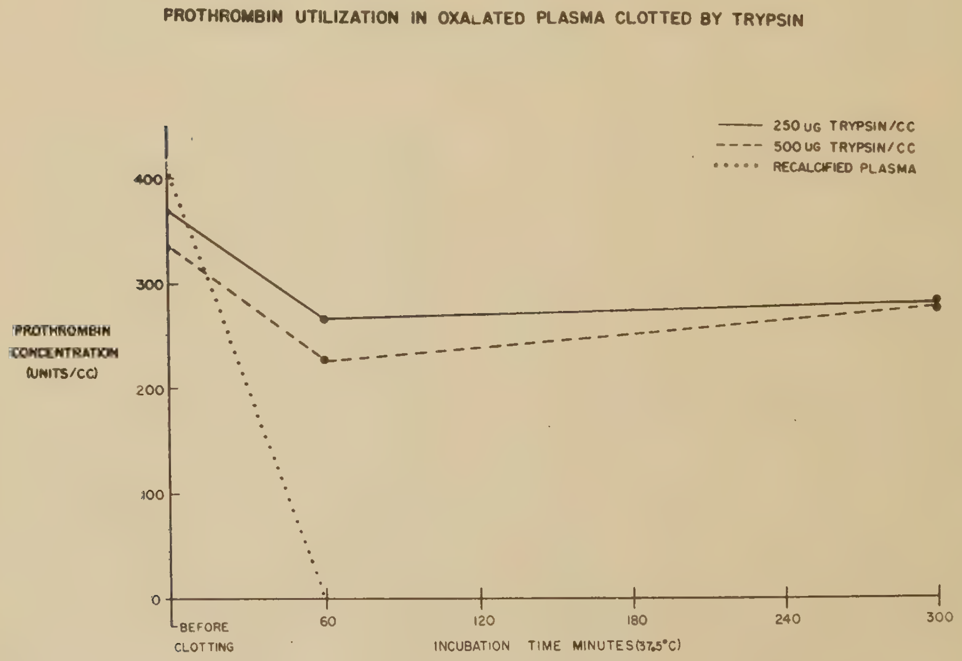

FIG. 3. Prothrombin utilization in trypsin-clotted oxalated plasma. 1 ml samples of oxal. plasma were clotted by adding 0.1 ml of 0.85% sal. containing 250 and 500 μg trypsin. For control, 1 ml plasma was clotted by adding 0.2 ml 2% $CaCl_2$ and 0.3 ml of thromboplastin (Difco). The samples were inc. at 37.5°C and prior to assaying for proth. the clots were removed and 0.1 ml trypsin inhibitor (400 μg) in 0.85% sal. was added to the supernatant plasma.

TABLE I. Inhibitory Effect of Heparin on Trypsin's Clotting Action. Plasma was prepared by diluting 7 pt normal human blood with 1 pt 1.85% potass. oxal. in distilled H_2O. The oxal. blood was then centrifuged at 2500 rpm for 30 min. and the plasma pipetted off. Reagents were kept at 37.5°C and mixed in the order listed in the table. Trypsin was added in sal. sol. in 0.2 ml amounts. Hep. was added as 0.01 ml of liquid hep. 1 mg of protamine was added as 0.1 ml 1% protamine sulf. sol. Controls, consisting of 1 ml oxal. plasma, + 0.2 ml oxal. sal., 0.5 mg hep. and 1 mg prot., did not clot. The clotting effect of trypsin was not inhibited by 1 mg prot.

Reagents (mixed in listed order)			Clotting time in sec. (37.5°C)
1 ml oxalated plasma	+ 250 μg trypsin		35.7
" " "	+ 500 μg "		23.2
" " "	+ 0.5 mg heparin	+ 250 μg trypsin	No clot
" " "	" "	+ 500 μg "	"
" " "	" "	+ 250 μg " + 1 mg protamine	"
" " "	" "	+ 500 μg trypsin + 1 mg protamine	"
" " "	" "	+ 1 mg protamine + 500 μg trypsin	20.4

might be expected, the rate of prothrombin conversion paralleled the rate of thrombin formation (Fig. 1). However, it should be noted that not all the prothrombin was converted and a constant amount remained despite continued exposure to trypsin. Furthermore, the amount of unconverted prothrombin was the same for the various trypsin concentrations studied (7.5, 15, 20, and 50 μg/ml). Even the presence of calcium and labile and stable factors failed to alter the amount of residual prothrombin. Since about 25 of the original 85 units of prothrombin/ml remained, presumably about 60 units/ml of thrombin were formed. However, antithrombic impurities in the reagents, and the antithrombic effect of trypsin itself(15), prevented complete recovery of the thrombin.

To determine if trypsin required a plasma co-factor for the activation of prothrombin, trypsin was added directly to oxalated human plasma. However, it was again found that only a portion of the available prothrombin was converted (Fig. 3). From the graphs it appears as if a rise in prothrombin concentration occurred at 5 hours of incubation. However, the difference in concentration between the one- and 5-hour samples is within the limits of experimental error.

Other workers(16) have demonstrated that heparin abolishes the clotting effect of trypsin on oxalated plasma. This effect was investigated. The results (Table I) show that concentrations of trypsin which clot oxalated plasma do not clot the same plasma if a small amount of heparin is added prior to addition of trypsin. The addition of protamine to the plasma after addition of trypsin does not reverse the anti-coagulant effect of heparin. However, if protamine is added to the heparinized oxalated plasma prior to addition of trypsin, clotting proceeds as usual. In order to clarify the mechanism of this effect, prothrombin utilization was determined after addition of trypsin. The results (Fig. 4) show that, although no clotting occurs, the reduction in prothrombin levels parallels that observed in ordinary oxalated plasma clotted by trypsin. The trypsin had converted a portion of the prothrombin but no clot formed. Presumably, the heparin raises the antithrombic activity of the plasma to levels sufficient to prevent the newly formed thrombin from being effective. That the heparin was sufficiently antithrombic to have inactivated the thrombin formed through trypsin's action on prothrombin is shown in Fig. 5.

Discussion. These experiments demonstrate that trypsin alone can convert prothrombin into thrombin. In this respect, the work of Eagle and Harris(4) is substantiated. However, it is shown that prothrombin is only partially converted into thrombin by trypsin. Furthermore, calcium and the "accessory

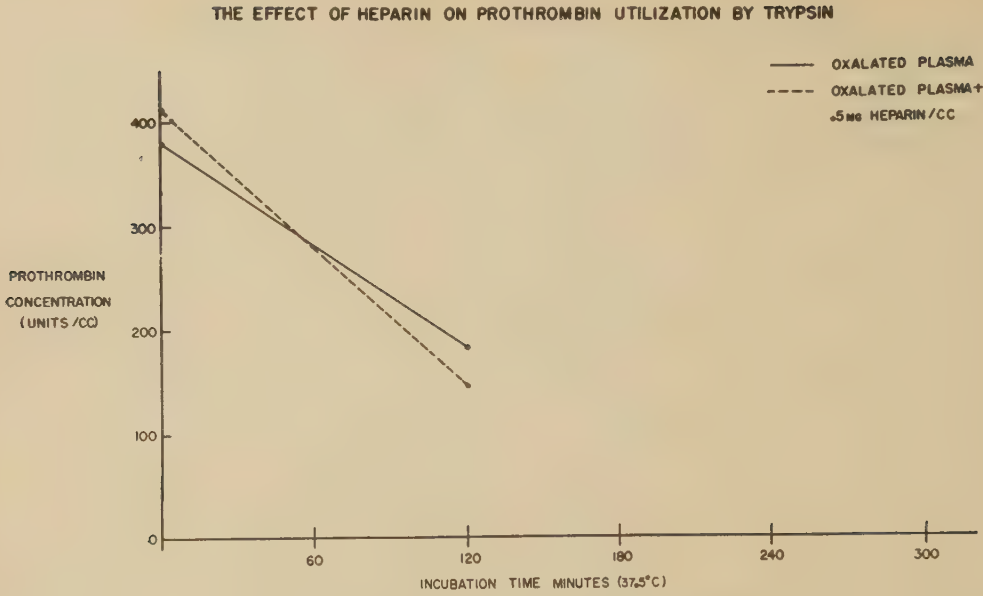

FIG. 4. Prothrombin utilization in heparinized, oxalated plasma.

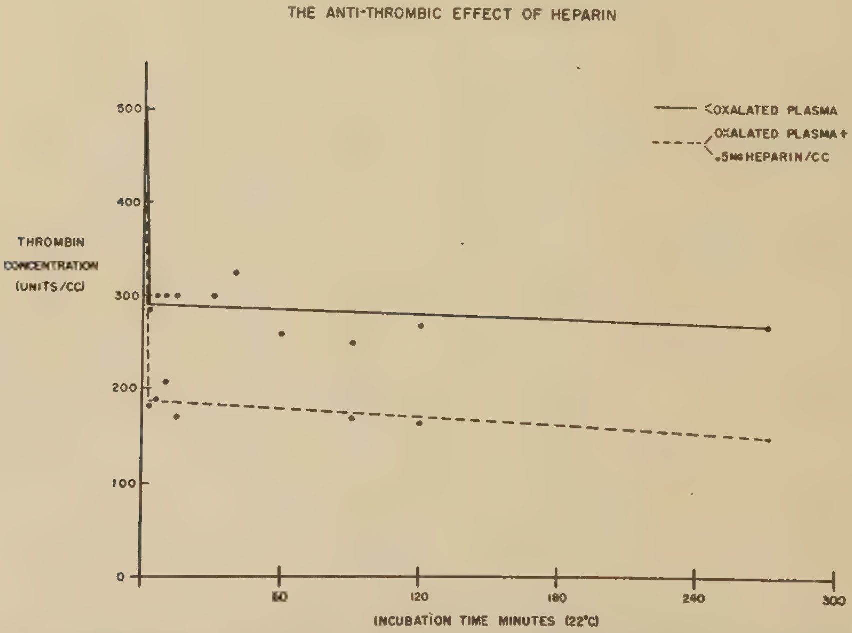

FIG. 5. Antithrombic effect of heparin. 5000 units of thrombin (Parke, Davis & Co.), in 10 ml 0.85% sal., was added to 10 ml oxal. plasma containing 0.5 mg heparin/ml. For control, the same amt of thrombin was added to an equal vol of oxal. plasma without heparin.

factors" are unnecessary for trypsin's action, although calcium does increase the rate of thrombin formation. This acceleratory effect of calcium on the reaction may have led earlier workers to believe that optimal amounts of thrombin were obtained only in the presence of calcium.

It must be emphasized that in the early experiments by other investigators, no attempt was made to inactivate the trypsin before determining the thrombin titer. Thus, the trypsin was free to act on prothrombin,

thrombin and fibrinogen. It was impossible, therefore, to determine which factor the trypsin was affecting. It contrast to these earlier experiments, the overlapping effect of trypsin was avoided by adding a trypsin inhibitor to the sample used for the thrombin assay.

It can also be added that we found no evidence for Lenggenhager's concept(8) that a plasma factor other than prothrombin itself is necessary for the clotting action of trypsin. Nevertheless, there still exists the possibility that our prothrombin was not freed of this hypothetical factor, and we are therefore reluctant to exclude the idea completely.

Because of the recent clinical use of trypsin intravenously, its clotting action has received considerable attention. Tagnon(5) investigated the use of heparin to counteract the intravascular clotting action of trypsin. Working with dogs and rabbits, Tagnon found that the previous injection of one mg/kg of heparin increased the minimal amount of injected trypsin needed to produce intravascular clotting. Other workers(16,17) have shown that heparin inactivates the proteolytic activity of trypsin, the degree of inactivation varying with the concentration of heparin and the incubation time. In this respect, the present experiments demonstrate that concentrations of heparin too low to inactivate the proteolytic activity of trypsin, may still inhibit the clotting action of trypsin. Heparin does this by raising plasma antithrombic activity, thus preventing the thrombin formed from trypsin's action from being effective. The effect observed by Tagnon was due, in all probability to increase antithrombic activity in the blood stream, and not due to direct inactivation of the trypsin.

Summary. 1. Trypsin converts prothrombin into thrombin. Calcium and accessory clotting factors are not essential. Calcium does, however, accelerate the rate of thrombin formation but without affecting the thrombin yield. 2. Conversion of prothrombin by trypsin is incomplete. 3. Heparin, in low concentrations, can inhibit the clotting effect of trypsin by increasing plasma antithrombic activity.

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Received September 25, 1953. P.S.E.B.M., 1953, v84.

Placental Esterases.* (20671)

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Since the placentas of man and animals lack innervation(1), it is of considerable interest that acetylcholine(2-4) and a cholinesterase (5) have been identified in human placentas. Torda found that cholinesterase activity of human placentas at full term approximated one tenth of that of pig's pyloric mucosa. Ord and Thompson(6) reported that homogenates of fresh placentas hydrolysed acetylcholine, benzoylcholine and acetyl β-methylcholine, but that homogenates prepared from perfused placentas possessed much less activity toward benzoylcholine. According to the criteria of Mendel, Mundell and Rudney(7), these results indicate that both acetylcholinesterase and serum cholinesterase are present in the placenta. Further evidence of the occurrence of cholinesterases was the inhibition of the enzymes by di-isopropyl fluorophosphate in a concentration similar to that required to inhibit the cholinesterases of brain. Wen, Chang and Wong(8) devised a method, which they believed demonstrated the activity of acetylcholine in tissue sections, by means of which they identified large amounts of acetylcholine in the syncytial trophoblast of the chorionic villi and in the intervillous space of the human placenta. With the recent development of several histochemical methods for the demonstration of esterases, it seemed advantageous to explore the placenta by these means and with the use of inhibitors to differentiate between various enzymes of the esterase groups. From an examination of a normal human placenta of 10 weeks' gestation age (9,10), it was found that with the method for esterase of Barrnett and Seligman(11) on unfixed frozen sections there is an intense reaction in the trophoblast clothing the chorionic villi, involving mainly the syncytium. With the esterase method of Nachlas and Seligman (12), however, carried out on sections of acetone-fixed, paraffin-embedded material, the trophoblast showed no reaction. From these observations, the conclusion was reached that the combination of a positive Barrnett and Seligman reaction on unfixed frozen sections, and of a negative reaction by the method of Nachlas and Seligman upon acetone-fixed sections, indicated the presence of a cholinesterase and possibly a small amount of aliesterase in the trophoblast.

The present paper describes the results of histochemical experiments on a human and a cat placenta in an attempt to determine by the use of inhibitors which esterolytic enzymes are present.

Material and methods. Small blocks of tissue were obtained from a placenta of a cat, near the middle of gestation (fetal crown-rump length: 7.2 cm). Other blocks were obtained from a normal human placenta of 10 weeks of gestation (fetal crown-rump length: 3.2 cm). From the human specimen pieces of both labyrinth and decidua were taken. Several blocks were placed in cold acetone (4°C) and then embedded in paraffin and sectioned. Other blocks were frozen and sectioned in a cryostat without any previous fixation. Deparaffinized sections of the acetone-fixed material were stained by the methods of Gomori (13) and Nachlas and Seligman(12). The method of Barrnett and Seligman(11), which employs indoxyl acetate as the chromogenic substrate, was applied to fresh frozen sections. Frozen sections were also stained by the method of Ravin, Zacks and Seligman(14), which employs 6-bromo-2-naphthyl acetate and tetrazotized diorthoanisidine. The method of Ravin, Tsou and Seligman(15) was employed to detect serum cholinesterase activity by means of a specific substrate; carbonaphthoxycholine iodide and tetrazotized diorthoanisidine. Frozen sections were also pretreated for one hour in barbital buffer (.1 M at pH 7.5) containing physostigmine salicylate (3.1 x 10^{-5} M or 6.2 x 10^{5} M)

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* This investigation was aided by a grant from the National Institutes of Health, U. S. Public Health Service.

† Jeffries Wyman Scholar, Harvard University, 1952-53.

or sodium taurocholate 10^{-3} M, and staining was carried out in the presence of these inhibitors to distinguish cholinesterase from aliesterase and lipase. *Acetylcholinesterase* and serum *cholinesterase* are completely inhibited by low (10^{-5} M) concentrations of physostigmine(16,17), but aliesterase is somewhat inhibited when a concentration of 10^{-3} M is reached. The activity of both cholinesterases is completely destroyed by cold acetone, whereas the activity of lipase and aliesterase is at best 40-60% reduced by this treatment(12). The activity of serum cholinesterase can be distinguished from acetylcholinesterase, since only the former enzyme is able to hydrolyse the chromogenic substrate carbonaphthoxycholine iodide. Lipase is either activated or unaffected by sodium taurocholate 10^{-3} M (18), while aliesterase is inhibited to a variable but usually significant degree. Thus, this combination of reactions permits a differentiation of cholinesterases, on the one hand, from aliesterase and lipase on the other. An esterase which hydrolyses 6-bromo-2-naphthyl acetate and is inhibited by physostigmine (10^{-5} M) and cold acetone is regarded as a cholinesterase, but esterases which resist these inhibitors may be aliesterase, lipase or a mixture of both. Lipase can usually be distinguished by its activation or lack of inhibition by sodium taurocholate, and aliesterase, by its inhibition with this reagent.

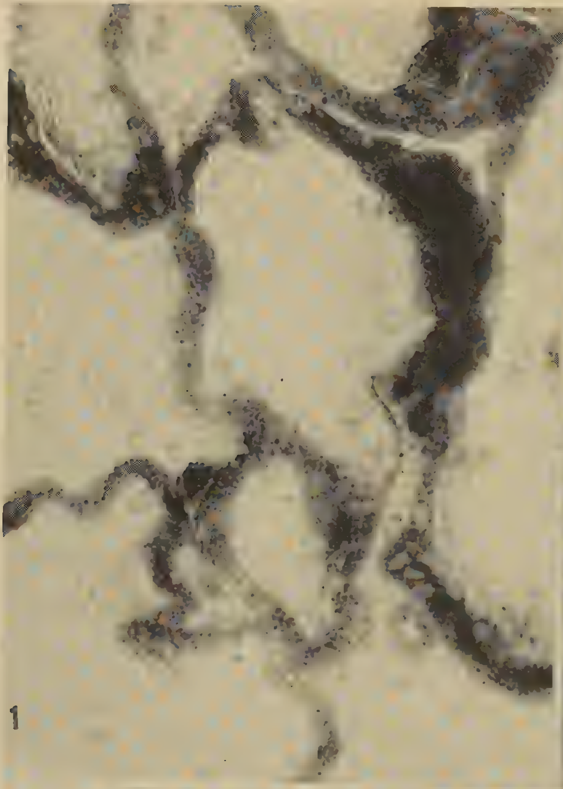
Observations. Cat's placenta. After 20 minutes' incubation in 6-bromo-2-naphthyl acetate and tetrazotized diorthoanisidine(14) the epithelium of the subplacental uterine glands was filled with punctate, blue granules (Fig. 1), and a less intense reaction was present in the trophoblastic lamellae of the chorioallantoic placenta. Sodium taurocholate or physostigmine salicylate 3.1 x 10^{-5} M slightly inhibited the enzymatic activity in the trophoblast. The placenta and uterine glands failed to stain with carbonaphthoxycholine iodide and tetrazotized diorthoanisidine(15), a result which indicated the absence of serum cholinesterase. Acetone-fixed, deparaffinized sections stained by Gomori's procedure showed an intense reaction of the subplacental uterine glands, a milder reaction of the trophoblast facing the glands and no reaction of the lamellae of the placental labyrinth. The characteristic columnar cells of the brown border exhibited a variably strong reaction (Fig. 2), whereas the paraplacental uterine mucosa, including the surface epithelium and glands, was negative. The brown border was examined only in paraffin sections.

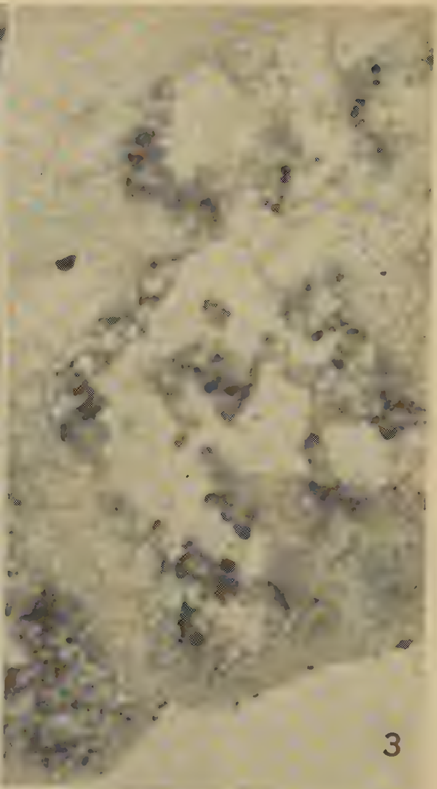
Human placenta. Frozen sections of the labyrinth of the human placenta, incubated for 20 minutes in a mixture of 6-bromo-2-naphthyl acetate, showed dense deposits of blue granules in the trophoblastic syncytium and lesser amounts in the Langhans cells (Fig. 3). The stroma of the chorionic villi, including the Hofbauer cells, did not react. The cells lining the uterine glands and the epithelium covering the decidua were somewhat less intensely reactive. Decidual cells of two types were observed; the larger of these were unreactive but the smaller contained some dye granules indicative of enzymatic activity. The large trophoblastic cells of the cell columns and basal plate were unreactive (Fig. 4). The uterine stroma and musculature appeared to be negative. Physostigmine salicylate 3.1 x 10^{-5} M slightly inhibited the enzymatic activity.

The method of Ravin, Tsou, and Seligman for serum cholinesterase was negative in all regions of the placenta. Acetone fixed, deparaffinized sections of placenta, stained by the esterase method of Nachlas and Seligman, were also completely negative.

Discussion. Since the enzymatic activity in the trophoblast and uterine glands of the cat's placenta is slightly inhibited by physostigmine and cold acetone and is not activated by sodium taurocholate, it is chiefly due to an aliesterase. The slight inhibition when physostigmine is used may be attributable to a small amount of acetylcholinesterase activity.

The esterase in human trophoblast and uterine glands behaves differently than acetylcholinesterase or aliesterase in other tissues. For example, the acetylcholinesterase activity of motor end plates is completely inhibited by cold acetone and 10^{-5} M physostigmine, whereas the activity of human placental esterase is abolished by cold acetone but only slightly inhibited by physostigmine. From the result of acetone fixation, it would appear that a





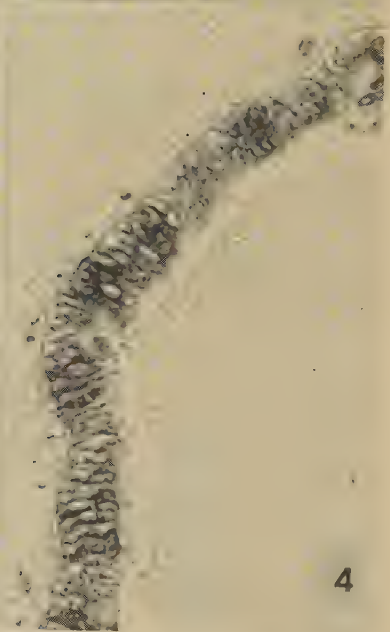


FIG. 1. Human placenta at 10 wk gestation showing intense esterase activity of chorionic villi by the method of Ravin, Zacks and Seligman. The granular reaction product is localized almost exclusively in the trophoblast (placental membrane) covering the villi. The chorionic stroma is negative. × 350.

FIG. 2. A portion of the same human placenta showing a secondary chorionic villus attached

to a trophoblastic cell column. The trophoblast of the secondary villus (left) reacts intensely for esterase, whereas the cell column is negative. × 350.

FIG. 3. Subplacental uterine gland of a cat, showing intense esterase activity (method of Ravin, Zacks and Seligman). × 250.

FIG. 4. The brown border of the membranous chorion of a cat showing a strong reaction for esterase in the columnar trophoblast (Gomori's method). The uterine epithelium opposite columnar cells is negative, as are also stroma and paraplacental glands of uterine mucosa. × 200.

cholinesterase is responsible for the esterase activity of human placenta, although the failure of physostigmine to inhibit this activity presents an unexpected difficulty. This apparent inconsistency may be explained either by the presence of low placental aliesterase activity which is inhibited completely by cold acetone, or by a cholinesterase which is resistant to low concentrations of physostigmine. The latter possibility gains added weight from the evidence of the presence of acetylcholinesterase and serum cholinesterase in the human placenta(6). Similar apparent inconsistencies have been noted in various tissues of the rat(19,20).

It is of interest that the esterase of human placenta differs from the esterase found in the cat's placenta. The cat's placental enzyme is presumably aliesterase, whereas the human placental enzyme is possibly a cholinesterase. The functions of esterases in the placenta are obscure. Since the organ lacks innervation, there is no apparent need for acetylcholinesterase which functions in synaptic transmission. One can only surmise that the esterases located in the trophoblast probably play a role in the transfer of substances across the placental barrier.

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Received October 5, 1953. P.S.E.B.M., 1953, v84.

Fate of Antibody Following *in vivo* Combination with Specific Antigen.* (20672)

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It is well known that the level of circulating antibody falls abruptly after the injection of specific antigen. This decrease in circulating antibody is presumably the result of the formation of antibody—antigen complexes which are quickly removed from the blood. The fate of the antibody following its combination with antigen and removal from the blood has not been described. In a previous study we traced the fate of an I^{131} labelled protein antigen during and following its *in vivo* combination with antibody(1). It was found that the antigen was catabolized rapidly following its elimination from the blood. The present report deals with the *in vivo* fate of the antibody portion of the immunologic complex.

We have labelled the gamma globulin fraction of rabbit antiserum to bovine albumin with I^{131} and traced the fate of the labelled antiserum after its passive administration into rabbits, some of which contained the specific antigen. I^{131} labelled proteins retain their radioactive label until they are degraded beyond the point of immunologic recognition, at which time the I^{131} appears in non-protein bound form, chiefly as iodide, and is excreted primarily by the kidneys(2). Therefore, the originally labelled homologous protein can be traced by determination of protein bound I^{131}, which exists in dynamic equilibrium in the intravascular and extravascular components of the plasma protein pool(3). The amount of protein catabolized can be determined from the measurement of non-protein bound I^{131} present in and excreted by the host.

Materials and methods. The gamma globulin fraction of pooled sera from rabbits repeatedly immunized with crystalline bovine serum albumin (BSA) (Armour and Co. lot No. 20) was obtained by ammonium sulfate precipitation(4). This gamma globulin fraction, containing 52 mg protein and 27 mg anti BSA antibody per ml, as measured by quantitative total and antibody nitrogen determinations(5), was labelled with I^{131} as previously described(6). The I^{131} labelled gamma globulin preparation (I*RGG) contained less than an iodine atom per molecule of protein, and approximately 99% of the I^{131} was protein bound as determined by trichloracetic acid precipitation. As has been previously reported, the degree of iodination of antibody molecules does not differ from that of other globulin molecules(7a,b,c). Therefore, it may be assumed that the I^{131}: protein ratio in the I*RGG was the same for both the specific anti BSA antibody molecules and the other globulin molecules. The anti BSA N : BSA N ratio at equivalence was approximately 5.5. Twelve young male albino rabbits weighing from 2.2 to 2.4 kg made up the 3 experimental groups used in this study. All were given non-radioactive iodide in their drinking water for several days prior to the injection of I*RGG in order to saturate iodine utilizing tissues and reduce non-specific retention of I^{131}. Rabbits were kept in individual metabolism cages for the collection of urine. Bladder urine was obtained by catheter and was combined with urine collected in the metabolism cage at each determination. The total amount of non-protein bound I^{131} in the body was calculated by multiplying the amount in 1 cc of whole blood by 37% of the body weight expressed in grams(1). Technics of protein bound I^{131} determination of plasma have been previously described(6).

*Group I: I*RGG.* Four rabbits each received intravenously a 2 cc injection of I*RGG containing 20 mg anti BSA antibody. Determinations of the protein bound I^{131} in the plasma and the non-protein bound I^{131} in total body and urine were made 1, 2, 3, 4, and 7 days after injection. *Group II: I*RGG followed by BSA.* Four rabbits each were in-

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* This study was supported by Atomic Energy Commission. This is No. 29 of the Pathology Department.

jected intravenously with 4cc I*RGG containing 40 mg anti BSA antibody. Forty-eight hours later, immediately following determination of protein bound and non-protein bound I^{131}, they received an intravenous injection of 50 mg BSA. Protein bound I^{131} determinations of the plasma were made 1 hour, 6 hours, 1 day, 2 days, and 5 days after injection of BSA. Nonprotein bound I^{131} determinations of body and urine were made 6 hours, 1 day, 2 days, and 5 days after BSA. *Group III*: *BSA followed by I*RGG*. Four rabbits each were injected intravenously with 40 mg BSA and 24 hours later were injected intravenously with 5 cc I*RGG containing 50 mg anti BSA antibody. Protein bound I^{131} determinations of the plasma were made 1 hour, 6 hours, 1 day, 2 days, and 3 days after the I*RGG. Non-protein bound I^{131} in body and urine were determined 6 hours, 1 day, 2 days, 3 days, and 6 days after the I*RGG.

Results. The amount of I*RGG in total plasma volume of each rabbit throughout the period of observation is presented in Fig. 1. The total accumulative non-protein bound I^{131} found in urine and body for each rabbit is shown in Fig. 2.

Group I: *I*RGG*. In the animals receiving only antiserum, the I*RGG disappeared from the plasma rapidly during the first 2 days and then at a much slower rate for the remainder of the period of observation (Fig. 1). The initial rapid disappearance from the blood is caused in large part by equilibration of the labelled protein between the intra- and extravascular components of the plasma protein pool(8). The second, slower rate of elimination is the result of normal metabolic degradation of gamma globulin(9). The amount of non-protein bound I^{131} appearing during the first post injection day was greater than that appearing during subsequent days (Fig. 2). This difference was probably the result of rapid catabolism of any slightly altered protein and rapid excretion of the non-protein bound I^{131} in the I*RGG. The Group I rates of I*RGG elimination from the blood and its catabolism, as indicated by appearance of non-protein bound I^{131}, can be used as controls for comparison with the observations on Groups II and III, which received BSA in addition to I*RGG.

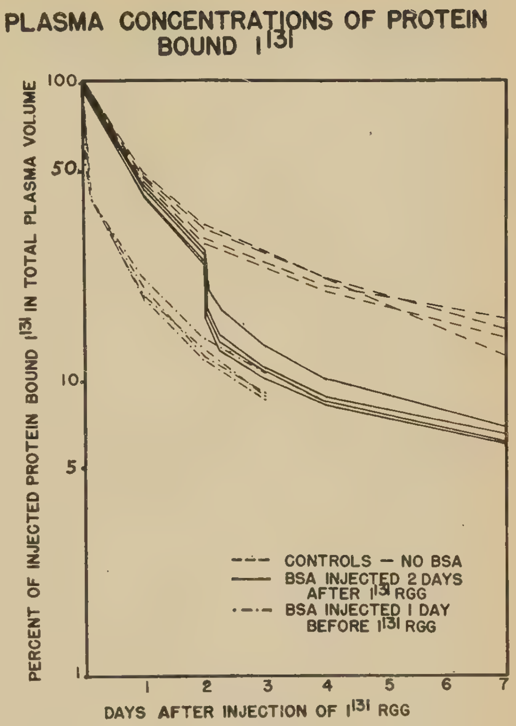

FIG. 1. % of injected I*RGG in total plasma vol. Individual line for each rabbit indicates values during course of observations. In Group II (solid lines) the administration of BSA on Day 2 is followed by a prompt drop in plasma I*RGG values to about one-half those seen in Group I controls and to the range of those of Group III.

Group II: *I*RGG followed by BSA*. This part of the experiment was designed to observe the effect of an excess of antigen on the fate of antibody which had equilibrated within the plasma protein pool, thereby having a distribution somewhat comparable to actively produced antibody. In these animals the blood levels of I*RGG and the amount of non-protein bound I^{131} two days after injection of I*RGG and immediately prior to the injection of BSA were similar to those found in the controls, as would be expected. However, within one hour after the injection of BSA, the I*RGG levels in the plasma fell from an average of 25% of the original injection to 17.7%, within 6 hours to 14.3 per cent, and within 24 hours to 11% (Fig. 1). Twenty-four hours after injection of BSA, the amount of I*RGG in the blood was approximately

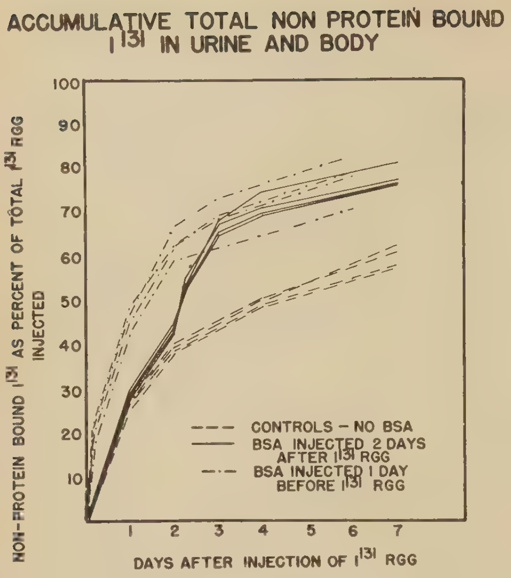

FIG. 2. Rate of appearance of non-protein bound I^{131} indicating catabolism of I*RGG. In Group II after injection of BSA the total non-protein bound I^{131} increases rapidly from values near those of the Group I controls to values comparable to those of Group III.

one-half of that expected without injection of BSA, according to Group I values. The rate of elimination of I*RGG from the blood during the one to 5 day period after injection of BSA closely paralleled the final rate of elimination in Group I. Coincident with the rapid loss of I*RGG from the plasma after injection of BSA, there was an increase in the rate of appearance of non-protein bound I^{131} (Fig. 2). During the first 6 hours after the BSA injection, almost 10% of the injected I^{131} appeared in the non-protein bound form in excess of the amount expected had no BSA been given, and during the first 24 hours this excess had increased from 15 to 20% of the original injection. The rate of appearance of non-protein-bound I^{131} fell off abruptly the second day following injection of BSA.

*Group III: BSA followed by I*RGG.* This group was injected to see whether there would be any difference in the elimination and catabolism of antibody when the order of antibody and antigen administration was reversed. As can be seen in Fig. 1, there was a rapid loss of I*RGG from the blood immediately after its injection in the presence of BSA. From one third to one fourth of the I*RGG disappeared from the plasma within 5 minutes, almost one half within one hour, 60% within 5 hours, and 80% within one day. The amount of I*RGG in the plasma one and 2 days after injection was slightly less than one half that found in other groups, indicating specific elimination of a little more than one half of the I*RGG as a result of the presence of BSA. Three days after injection of I*RGG, the plasma protein bound I^{131} levels in Group III were comparable to those in Group II one day after injection of BSA. Non-protein bound I^{131} rapidly appeared in this group after injection of I*RGG. Within one day 45% of the injected activity was detectable in the non-protein bound form. The amounts of non-protein bound I^{131} found in Groups II and III from the third day on, after both had received BSA, were similar.

Discussion. Since the gamma globulin preparation, I*RGG, was approximately one half specific anti BSA antibody it would be expected that only one half, *i.e.,* the antibody, would be affected by the presence of BSA. As demonstrated by Groups II and III in Fig. 1, approximately one half the I*RGG was rapidly eliminated from the circulation in the presence of an excess of BSA. In Group II, within one hour after the injection of BSA, 31% of the I*RGG estimated to be circulating had BSA not been given was eliminated from the circulation; within 6 hours, 43% was eliminated; and within one day, 50% was eliminated. It seems likely that the 50% of the I*RGG eliminated in the presence of BSA was the specific anti BSA antibody. Assuming that were so, the percentage of anti-BSA antibody eliminated would be twice the above figures for I*RGG elimination, since specific anti BSA antibody made up approximately one half the total I*RGG protein. Since the intravascular and extravascular plasma proteins are in dynamic equilibrium, not only would one half of the I*RGG be removed from the plasma, but actually one half of the I*RGG in the entire body would be removed from the body's protein pool. The elimination rate of the remaining, non-antibody, I*RGG in Group II 24 hours or more after BSA injection agreed well with the observed rates of gamma globulin catabolism uncomplicated by the presence of antigen(9).

In Group III I*RGG underwent simultaneous equilibration and removal by virtue of the presence of BSA. Since the early rates of equilibration were not determined in Groups I and II, it is not possible to estimate the rate of I*RGG elimination caused by BSA in the first 24 hours in Group III. However, that the BSA induced elimination of I*RGG was completed within 24 hours is indicated by the fact that the one day plasma levels of I*RGG in Group III were slightly less than one half those in Groups I and II. It appears that the order of administration of antigen and antibody does not noticeably affect the rate of elimination of antibody from the circulation.

Associated with the elimination of anti BSA antibody from the circulation in the presence of BSA, there is an increase in the rate of appearance of non-protein bound I^{131} indicating an increase in the rate of catabolism of the I*RGG, presumably the anti BSA antibody. However, calculation of the exact rates of antibody catabolism is complicated by the fact that recovery of non-protein bound I^{131} was not complete. Since some non-protein bound I^{131} is eliminated in the breath and the feces and since collection of urine in metabolism cages is never complete, loss of some of the non-protein bound I^{131} is to be expected. The per cent of non-protein bound I^{131} lost can be determined by adding to the total protein bound I^{131} in the animal (approximately twice that in the plasma) the non-protein bound I^{131} present in and excreted by the host and subtracting the sum from 100%. Such calculations show the efficiency of our non-protein bound I^{131} collection for the 7-day period for Groups I and II and a 3-day period for Group III to be: Group I—87%, Group II—89%, and Group III—84%. There is little retention of non-protein bound I^{131} in pre-iodinated rabbits(8). If the observed Group II non-protein bound I^{131} values given on Fig. 2 are corrected for the 11% loss of excreted non-protein bound I^{131}, the increased accumulation of non-protein bound I^{131} expressed as per cent of anti BSA antibody theoretically present in the host had no BSA been given, was as follows: at 6 hours 39%, 24 hours 84%, and 48 hours 97%. These values, while admittedly only approximations, indicate that antibody catabolism is largely completed less than 24 hours after antibody is removed from the circulation as a result of the presence of circulating specific antigen.

If the non-protein bound values for Group III (Fig. 2) are corrected for the 16 per cent loss of non-protein bound I^{131}, the increased accumulation of non-protein bound I^{131} expressed as per cent of anti BSA antibody theoretically present in the host had no BSA been given, was as follows: at 24 hours, 67% and at 48 hours, approximately 100%. The observations on Group III are in general agreement with those of Group II and support the conclusion that the catabolism of antibody occurs rapidly after its elimination from the circulation.

The rapid rate of catabolism of antibody after its elimination from the circulation, presumably as part of an antigen—antibody complex or precipitate, is comparable to the rate of heterologous serum protein antigen catabolism under similar circumstances, as previously observed(1). This is in line with the observation that the rates of catabolism of homologous and heterologous serum proteins in the absence of an immune response are similar(8). It appears that the rabbit is equally capable of catabolizing foreign and native serum proteins and does not make a marked distinction between the two as far as rate of catabolism is concerned.

Pressman *et al.*, found that I^{131} labelled rabbit anti mouse kidney antibody, when localized in the glomeruli of mice, had a half-life of 20 to 21 days(10). The difference between the rate of loss of the antibody presumably fixed to the antigenic constituents of the glomeruli and the rate of degradation of antibody removed from the blood in the presence of circulating antigen might be in part related to the difference in species of host but more likely is the result of localization of the antibody. The antibody as part of a circulating antibody-antigen complex or precipitate would be taken up, in all likelihood, by reticuloendothelial cells which apparently have the ability to degrade protein rapidly.

The antibody fixed to the antigenic components of the glomeruli might not be exposed to cells with such proteolytic powers. If the antibody were attached to the glomerular basement membrane, which has been shown to be the most antigenic of the kidney's components(11), it might even be extracellular and therefore not exposed to proteolytic attack by intracellular enzymes.

Conclusions. In the presence of specific antigen 62% of the homologous anti-protein antibodies are rapidly removed from the circulation within one hour after injection and 86 per cent within 6 hours. Once removed from the blood, the antibodies are promptly catabolized, between one third and one half within 6 hours and virtually all between 24 and 48 hours. In the rabbit the rates of catabolism of anti protein antibodies and heterologous serum protein antigens after their elimination from the circulation as specific complexes are comparable.

Since the submission of this manuscript for publication a paper dealing with this subject by S. P. Masouredis, L. R. Melcher and M. B. Shimkin entitled "The Effect of Antigen Administration on the Behavior of I^{131} Labelled Rabbit Anti-Ovalbumin in Mice" has appeared in the *Journal of Immunology,* 1953, v71, 268.

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Received October 13, 1953. P.S.E.B.M., 1953, v84.

Reproducibility of X-Ray Survival Curves for Yeast Cells.* (20673)

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While investigating the influence of "modifying factors" (anoxia, protective chemicals, temperature, and physical state) on radiosensitivity of yeasts(1), it was found that the changes in radiosensitivity that had to be measured were relatively small. This made it necessary to develop a technic that would yield accurate and reproducible X-ray survival curves and to investigate cultural and physical conditions that might influence the radiosensitivity of the cells and the shape of their survival curves. These investigations are described in this paper. The influence of other modifying factors will be reported elsewhere.

Methods. Unless otherwise specified, a haploid strain of the yeast *Saccharomyces cerevisiae* was used; in one experiment a genetically related diploid strain was used, both strains being supplied by Prof. R. E.

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* This research was partially supported by the U. S. Atomic Energy Commission and carried out in the laboratory of Professor R. E. Zirkle while the author was a Pre-Doctoral Fellow of the U. S. Public Health Service.

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Zirkle. Stock cultures on Difco potato dextrose agar (PD agar) slants were kept at —30°C. From these, monthly subcultures were prepared, incubated at room temperature (25°) for 4 days, and stored at 0°. Streak cultures were prepared from the subcultures daily and incubated at room temperature until used (14 days unless otherwise specified). Cells were harvested with a wire loop and suspended in M/15 KH_2PO_4 at a concentration of approximately 10^7 cells/ml. (Acid phosphate was used for all suspensions and dilutions.) After agitation the cells were washed by 3 centrifugations (2000 g) of 5, 1, and 5 minutes, respectively. The cells were resuspended in an Erlenmeyer flask and placed on a Boerner rotary shaker for 90 minutes, after which they were filtered through a 5-cm thickness of pyrex wool to remove large pieces of debris and clumps of cells that might be present. The cells were examined microscopically for clumping or budding, and a hemocytometer was used to count the number of cells/ml. After dilution to 10^6 cells/ml, approximately 200 ml of this suspension in a 500-ml flask were shaken for at least 3 hours. Samples were then taken from this stock suspension as needed. The X-ray machine was a General Electric Maximar, operated at 250 KV, 15 ma, with inherent filtration only. The exposure rate was 425 ± 5 roentgens/minute at the surface of the irradiated suspensions, as read on a Victoreen chamber placed in the same plane as the yeast suspensions. For irradiation, 1.2-ml samples from the stock suspension were placed in plastic-capped glass vials, 21 mm in diameter and 50 mm high. Not more than 3% of the X-ray beam was absorbed in traversing the yeast suspension. Uniform doses were attained by rotating the vials (80 rpm) in a horizontal plane 18 cm from the cone aperture of the X-ray machine in such a manner that the bottom halves of the vials were submerged in a bath kept at 20.0 ± 0.1°C. Control samples were kept in the same bath and were shielded with lead.

After the desired treatment (X-raying, etc.), each sample was diluted accurately, and 0.5 ml aliquots were spread with L-shaped glass rods on the surface of PD agar plates that had been poured 3 days earlier and allowed to dry. Dilutions were so made that 200 to 400 colonies would be formed per plate (except at very low survivals), a visible colony being taken as the criterion of a viable cell. Within one hour after plating (first buds appeared in 2 to 4 hours) plates were incubated at 30° ± 1° for 64 hours without further movement. Visible colonies were then counted and surviving fractions computed as the ratio of the number of cells/ml surviving X-irradiation to the number of cells/ml in controls. Usually 2 vials were used per experimental point and 4 plates per vial. The computed standard errors of the means were only slightly larger than sampling errors when survival was greater than 0.01, but increased somewhat for survivals less than this. For several survival curves the experimental points were fitted to an exponential curve by the method of least squares. However, since fitting by eye gave results to within 0.5% of those obtained by least squares, most curves were fitted in this way.

Results. Curve A of Fig. 1 is a standard X-ray survival curve for haploid yeast obtained by the technic described above. The standard errors of the experimental points are less than 2% (approximately the size of the points of the graph) and hence the shape is reliably determined. The experimental points can be fitted by an exponential curve‡ with considerable precision. In several experiments with haploid yeast, slight departures from the exponential relation, $s = e^{-nkIt}$, were noticed at low doses. Curve B of Fig. 1 shows the experimental points obtained in the most divergent experiment. The solid line through the points is theoretically obtained by considering that there are 2 classes of cells present: 1) normal haploid cells, and 2) "divided haploid" cells (cells that might be in nuclear division or might have completed division without visible budding; see *Discus-*

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‡ Exponential survival curves are defined by the relation $s = e^{-nkIt}$; s is the surviving fraction of cells after a period of irradiation t minutes at a rate of I roentgens/minute, and n is the number of "sensitive sites" each of sensitivity k (see Zirkle and Tobias(6)). Thus nk is a direct measure of the radiosensitivity.

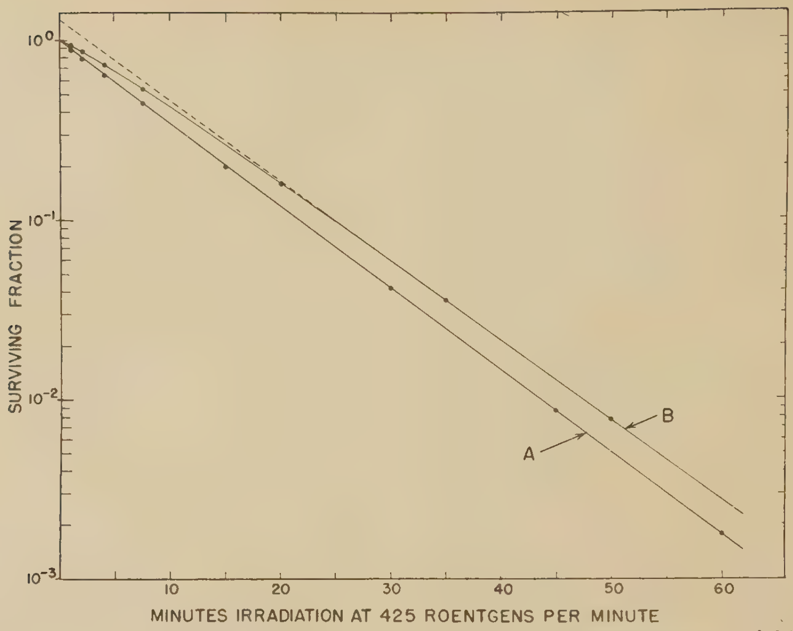

FIG. 1. X-ray survival curves of haploid yeast. Experimental points have errors of 2% or less (approximately the size of the points). Curve A: normal haploid survival, $s = e^{-0.106t}$ (t is irradiation time in min.). Curve B: contamination with "divided haploids," $s = 0.67e^{-0.103t} + 0.33\,(2e^{-0.103t} - e^{-0.206t})$.

sion). This analysis would also hold for the case of contamination with clumped or budded cells; microscopic examination, however, revealed neither. Furthermore, experiments showed that this departure from the exponential did not depend in a predictable fashion on the age of the culture from which cells were obtained. As but few haploid curves showed this small departure from the exponential, X-ray survival curves of the haploid yeast will be considered exponential.

TABLE I. Reproducibility of X-ray Survival Curves for Haploid Yeast.

Date	nk × 10^4 (roentgens^{-1})	Deviation (%)
9–13–51	2.36 ± .05	2
3–21–52	2.46 ± .04	2
7– 8	2.52 ± .05	4
8–10	2.46 ± .03	2
2–23–53	2.26 ± .06	6
Avg	2.41	

TABLE II. Dependence of Radiosensitivity of Haploid Yeast on Age of Culture.

Culture age (days)	Radiosensitivity, nk × 10^4 (roentgens^{-1})
2	3.58 ± .07
6	2.47 ± .05
14	2.41 ± .04
28	2.42 ± .04

That these results are reproducible over extended periods is shown by the data of Table I in which the radiosensitivities (nk) are given for typical experiments with 14-day cultures over a period of 18 months.

Several hours were often required for the completion of an experiment. However, aliquots of yeast suspension taken from a stock suspension (10^6 cells/ml) at various intervals up to 20 days showed no statistically significant change in either radiosensitivity or titer. Furthermore, cells irradiated at concentrations of 2.5 x 10^3 and 10^6 cells/ml displayed the same radiosensitivity although the titer of non-irradiated cells stored at the lower concentration dropped by a factor of 2 in 24 hours unless fortified with filtrate from a concentrated yeast suspension. Viability as used above is measured by colony formation from non-irradiated cells and does not necessarily mean that all cells in the controls are viable.

Microscopic examination, however, of

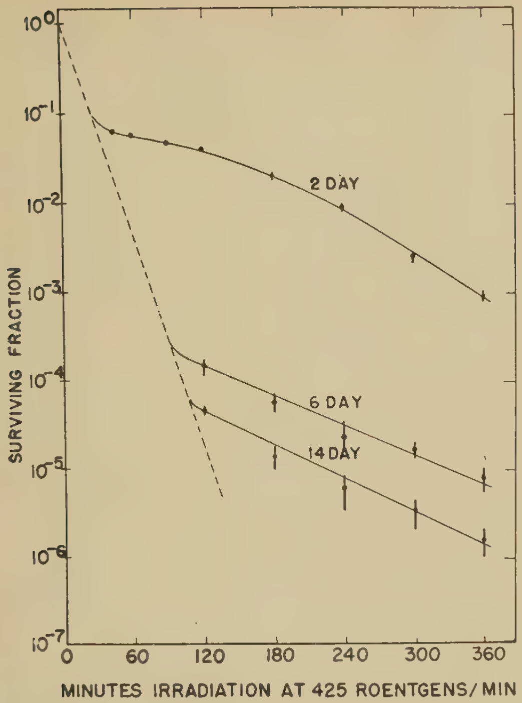

FIG. 2. "Tail" formation on x-ray survival curves as a function of age of culture. Dashed line represents initial exponential portion of each curve.

plated aliquots revealed that at least 98% of all cells prepared by means of the standard technic form visible colonies.

In Table II the results of a typical experiment are listed illustrating the dependence of the radiosensitivity, nk, on the age of the culture from which the stock suspension was prepared. Very young cultures (2-day) are considerably more sensitive than older ones. Often this is "explained" by the rule of Bergonie and Tribondeau(2) which states that the radiosensitivity of cells is directly related to their "activity"; generally young cells are considered more active. However, "starving" 2-day cells by agitation in acid phosphate for 48 hours before irradiation does not change their radiosensitivity although their activity should be reduced to a very low level by this treatment.

In Fig. 2 it can be seen that at high X-ray doses there is a definite break from the exponential part of the curve with the formation of a "tail" and that the older the culture (up to 14 days), the lower is the appearance of the tail on the curve. To test if the tail were caused by a genetically more resistant strain, X-ray survival curves were obtained for 32 cultures made from tail-survivors of a previous irradiation. In all cases the usual type curve was obtained.

To test if the tail position for young cultures could be varied, survival curves were obtained for a 2-day culture that had been "aged" by agitation in acid phosphate for 4, 24, and 48 hours. The point of origin of the tail remained remarkably constant although there was a small change in the shape and slope of the tail region, the curve for the more "aged" cells showing increased concavity downwards. Careful attempts to determine if there were any photoreactivation of X-ray inactivated yeast gave negative results. Latarjet(3) has reported an increased survival of X-rayed *Saccharomyces ellipsoideus* when stored in the cold before incubation. However, storage at 5° of X-rayed haploid yeast used in these experiments for periods up to 7 days before incubation gave decreased rather than enhanced survivals.

In Fig. 3 the experimental points of an X-ray survival curve are shown for the related diploid strain of yeast. The solid

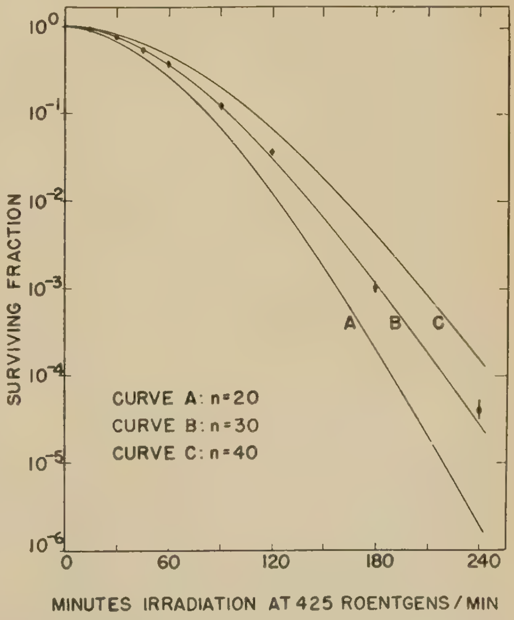

FIG. 3. X-ray survival points of diploid yeast. Curves A, B, and C are computed from the relation $s = (2e^{-kIt} - e^{-2kIt})^n$, with n given the various values shown.

curves were obtained from theoretical considerations that will be discussed later.

Discussion. It is desirable to place the test organisms in such a state that they form a homogeneous population. There are at least 2 ways to approach this. 1) Use a growing but statistically homogeneous population from a device such as a "chemostat"(4). 2) Use a resting population such as would be obtained if cells were kept on agar slants for long periods. Here it would be hoped that the cells would have approached a common, resting state. Cultures grown by the first method display considerable variation in the size of individual cells and there is a great deal of budding and clumping; these factors might easily influence the radiosensitivity. However, cells harvested from the surface of PD agar after 6 days or more of growth show no clumping, less than 0.1% budding, and are approximately uniform in size (4 to 5 μ in diameter). For these reasons cells were grown on agar slants.

Cells prepared as described in *Methods* appear to be in a steady state condition as determined by the consistencies in the radiosensitivity and in the titer. Hence time *per se* can be eliminated as a disturbing factor for suspensions of 10^6 cells/ml.

The individual experimental points of most published survival curves for various radiations have errors of 10% or more, and the curves themselves are not very reproducible. However, the above methods allow points of X-ray survival curves to be determined quite accurately; thus it is possible to ascertain the shape of the curves with considerable exactness. The normal haploid X-ray survival curve (Curve A of Fig. 1) for a 14-day or older culture is exponential down to survivals of 10^{-4} or less. This is interpreted as indicating that inactivation of haploid yeast by X-rays is due to a single event.

Occasional departures from the exponential type curve (Curve B of Fig. 1) do not necessarily conflict with the above interpretation. A possible explanation of Curve B is the following. In any population of yeast some of the cells might be in or might have completed nuclear division without visible bud formation. These divided haploids, having 2 sets of chromosomes, would be more resistant to irradiation. Consider the simplest possible case: a fraction f_2 of the population has undergone complete nuclear division without bud formation. Then the fraction $f_1 = 1 - f_2$ of the population is normal.[§] The survival of such a mixed population to X-rays would be given by $s = f_1e^{-nkIt} + f_2(2e^{-nkIt} - e^{-2nkIt})$. The slope nk can be obtained from the low survival region of the curve. An excellent fit is obtained (the solid line of Curve B, Fig. 1) by assuming that f_2, the fraction of divided haploids, is 0.33 ± 0.05 in this experiment.

No adequate explanation can be suggested for the dependence of the radiosensitivity on the culture age (Table II). Although growing, young cells might be more "active" than resting, old cells, a pretreatment similar to the one used here was shown by Aldous, Fisher, and Stern(5) to reduce carbon dioxide production and oxygen consumption to an endogenous level. Certainly the 48-hour agitation in acid phosphate of 2-day cultures should have changed the radiosensitivity (the initial slope) to that characteristic of older cultures if cellular activity alone determined the radiosensitivity. It is entirely possible that the "cross-sections" for the pertinent inactivation processes depend on the previous metabolic history of the cell. For example, it may be that older cells accumulate higher concentrations of cellular products that could serve as protecting agents against diffusing poisons produced by irradiation.

The tail effect (Fig. 2) has previously been reported by Zirkle and Tobias(6) and by Latarjet and Ephrussi(7), although it was observed at much higher survivals (around 0.05). Possible explanations for this difference are: 1) yeast were probably not placed in a state of endogenous metabolism; 2) irradiations were carried out on blocks of agar instead of in aqueous suspensions; 3) a different criterion of viability was used by Zirkle and Tobias (microscopic colonies of 3 or more cells instead of visible colony formation); 4) a different strain of yeast may have been used by Latarjet and Ephrussi. The fact that the "tail" regions are approximately exponential suggests that 2 populations are involved; the first (the larger) has the radiosensitivity characteristic of the initial part of the curve, the second, that of the tail region. This second fraction depends on the age of the culture. That these resistant cells are not genetically stable was revealed by the fact that cultures grown from them have the radiosensitivity characteristic of normal cells. From the experiments in which young cells were "aged" in acid phosphate, it appears that this resistant fraction remains constant under resting conditions.‖ It is interesting to note that while young cultures have a larger fraction of radioresistant cells, the rest of the cells are more sensitive than in older cultures (Table II).

[§] This is, of course, an oversimplification; there would be many intermediate cases.

Zirkle and Tobias(6) have estimated the number of "sensitive sites" in the haploid yeast necessary for continued cell division. It is worth while to repeat their calculations using these more accurate survival curves. The haploid curves provide data for determination of nk. The theoretical diploid X-ray survival curve is given by the expression $s = (2e^{-kIt} - e^{-2kIt})^n$, where the symbols are as before. In Fig. 3 the above function is plotted for assumed values for n of 20, 30, and 40, nk being obtained from the haploid curves. A very good fit to the experimental points is obtained for $n = 30$. Hence, under the given experimental conditions, inactivation of both members of any one of 30 ± 5 pairs of "sensitive sites" in the diploid cells results in inability to form visible colonies. Presumably this is also the number of "sensitive sites" in the haploid cell.

The fact that good exponential X-ray survival curves were obtained for the haploid strain down to survivals of 10^{-4} or less (for 14-day or older cultures) indicates that the method used here has certain advantages over those of Zirkle and Tobias and of Latarjet and Ephrussi. Also studies on effects due to temperature, protection, anoxia, etc., can be more easily carried out in aqueous suspension. The goodness of fit of the experimental points to an exponential curve is good evidence that the rate limiting process of inactivation is first order, *i.e.*, single event as postulated by Zirkle and Tobias in their diffusion model(6). Although the culture age has considerable influence on the radiosensitivity, exponential inactivation was observed for all ages. Other modifying agents have been used for this yeast system; Birge and Tobias(9) found yeast more resistant under anaerobic conditions; Wood(1) has investigated the effects of various temperatures and of phase state on the radiosensitivity. In all cases, although considerable changes in radiosensitivity were observed, the survival curves were exponential. These results are most easily explained by use of the diffusion model of Zirkle and Tobias and are good evidence that a sizeable fraction of the inactivation is due to indirect action.

Summary. Accurate and reproducible X-ray survival curves were obtained for both haploid and diploid yeast under carefully controlled conditions. The haploid curve is exponential down to survivals of 10^{-4} or lower for old cultures; the diploid curve is sigmoid and a good fit can be obtained by use of the proper multi-target model. The slope of the haploid survival curve (the radiosensitivity) depends on the culture age, young cultures being more sensitive. The haploid curves exhibit "tails" at low survival levels; the position of the tail is also dependent on the culture age, young cultures showing the tail at higher survival levels. The goodness of fit of the experimental points of the haploid yeast survival curves to an exponential curve is evidence that the rate-limiting inactivation step is first order as postulated in the diffusion model of Zirkle and Tobias.

The author would like to thank Professor R. E. Zirkle for research facilities and encouragement during this work.

‖ Beam, *et al.*,(8) have reported a correlation between the fraction of cells composing the "tail" and the number of cells with buds. They attribute the increased radioresistance of the buds to a duplication of nuclear material.

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Received October 16, 1953. P.S.E.B.M., 1953, v84.

Potentiation of Pteroylglutamic Acid by Ascorbic Acid in Anemia of Scurvy. (20674)

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The citrovorum factor (CF) is an active metabolite of pteroylglutamic acid (PGA) (1-3). CF replaces PGA in the nutrition of certain microorganisms(4,5) and animals (6,7), reverses aminopterin toxicity(2,6,8), and is effective in the treatment of nutritional macrocytic anemias in man(9-13). The conversion of PGA to CF by rat liver slices(14) or by resting cells of *S. faecalis* A(15) is enhanced by ascorbic acid. Excretion of CF in the urine following PGA injection is increased by ascorbic acid administration in normal man (16,17), whereas in adult human subjects with scurvy the ability to convert PGA to CF is impaired(18). CF is more effective than PGA in treating the megaloblastic anemia occurring in monkeys fed scorbutic diets low in PGA content(19,20). These observations suggest that ascorbic acid deficiency may precipitate certain nutritional anemias by limiting the conversion of PGA to CF. From studies on 2 patients with scurvy and anemia data are here presented which demonstrate a potentiation by ascorbic acid of the effect of PGA upon erythropoiesis.

Methods. Hemoglobin concentration, hematocrit, and red blood cell count were determined every 3 to 4 days by standard technics. Reticulocyte counts were done daily by the dry method(21), and at least 1,000 cells were counted for each determination. Buffy coat ascorbic acid levels were measured by the method of Butler and Cushman(22). The 24-hour urine outputs were collected and stored without preservative in a refrigerator; upon completion of each daily collection, aliquots were taken and stored at –10°C for assay. The PGA and CF activities of these urines were determined by microbiologic assays which utilize the growth response of *L. casei*(23) and of *L. citrovorum*(1,24), respectively. The levels of vit. B_{12} in the serum were assayed by a modification of the method of Ross which utilizes the growth response of *Euglena gracilis*(25). Serum iron concentrations were determined by the method of Kitzes *et al.*(26) employing o-phenanthroline.

Clinical observations. Case 1. A 42-year-old white male, gave an 8 months' history of inadequate food intake, particularly of ascorbic acid containing foods. On admission, he was weak, pale, and poorly nourished. He had a prominent perifollicular petechial rash over the arms, legs, and trunk, and several large ecchymoses on the posterior aspects of both legs. The tourniquet test was positive, but other coagulation studies, including bleeding time, clotting time, and clot retraction, were normal. Ascorbic acid was not present in the buffy coat of the venous blood. Hematologic studies revealed a macrocytic anemia with a hemoglobin of 6.6 g %, a hematocrit of 21.6 vol %, and a red cell count of 2060000/mm^3. The bone marrow aspirate contained a hyper-

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active immature erythroid series. No megaloblasts were seen. The serum iron level was 137 μg %, with a total iron-binding capacity of 382 μg %. The total serum B_{12} level was 344 μμg/ml, which is within the normal range for this laboratory of 532 ± 2 standard deviations of 322 μμg/ml.

Case 2. A 49-year-old white male, chronic alcoholic, entered the hospital with complaints of weakness, pallor, soreness of the mouth, and dysphagia. He gave a history of inadequate food intake for about 7 months prior to hospital admission. Physical examination revealed pallor, evidence of weight loss, cheilitis, and a smooth, pale tongue. There was a perifollicular petechial rash and a hyperkeratotic folliculosis over the extensor surfaces of the arms and legs, and over the abdomen. Perineal and scrotal ulcerations were present. The liver was enlarged. The tourniquet test was negative and other coagulation studies, including bleeding time, clotting time, and clot retraction, were normal. Ascorbic acid was absent from the buffy coat of the venous blood. Hematologic studies revealed a white blood cell count of 2100/mm^3, a platelet count of 81000/mm^3, and a macrocytic anemia with a hemoglobin concentration of 2.9 g %, a hematocrit of 9.2 vol %, and a red blood cell count of 710000/mm^3. The bone marrow aspirate contained a predominance of abnormal red and white blood cell precursors, including numerous megaloblasts. Free acid was present in the gastric juice. The serum iron concentration was 169 μg %, with a total iron-binding capacity of 281 μg %. The total serum B_{12} level was 193 μμg/ml. A bromsulfonphthalein retention of 40% was evident 45 minutes after injection, and there was a 4+ cephalin flocculation. X-ray studies of the upper gastro-intestinal tract revealed a hiatus hernia and a nutritional deficiency pattern of the small bowel. The oral glucose tolerance and stool fat contents[‡] during a period of controlled fat intake were normal. The patient was transfused with packed cells from 1 liter of blood at the start of the study.

Procedure. Shortly after admission both patients were placed on a diet which consisted of boiled milk, rice, crackers, sugar, coffee, and water, and which thus provided little, if any, ascorbic acid. After an initial control period both patients received daily intramuscular injections of PGA[§]: 125 and 250 μg for 10 and 12 days, respectively. In addition to the scorbutic diet and PGA, both patients were then given 1 g of ascorbic acid orally daily, for 9 and 10 days, respectively. In both patients, following the administration of ascorbic acid, the signs and symptoms of scurvy cleared and ascorbic acid appeared in the venous buffy coats. During the final period of study, PGA and ascorbic acid were continued in each patient, but a regular diet replaced the scorbutic diet.

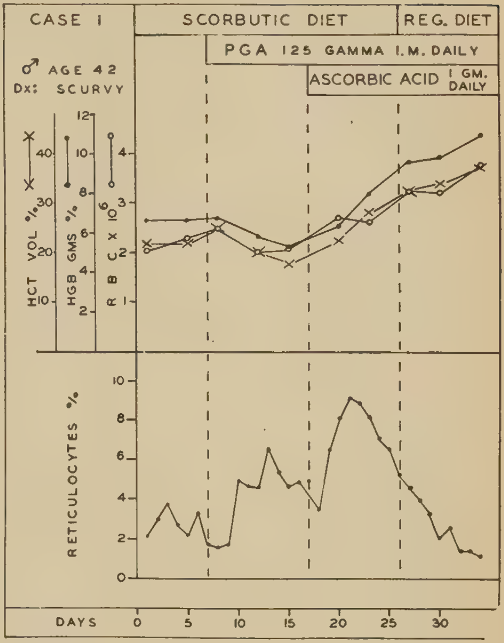

FIG. 1.

Results. When therapy with daily intramuscular PGA was instituted, a reticulocyte response occurred, with a maximum value of 6:5% on the seventh day. (Case 1, Fig. 1.) When ascorbic acid was given in addition to PGA and the scorbutic diet, a second reticulocyte response was observed which reached a maximum of 9% on the fifth day of combined therapy. The introduction of a regular diet produced no further response. The reticulo-

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[‡] Performed by Dr. Perry J. Culver.

[§] "Folvite" Solution, supplied by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

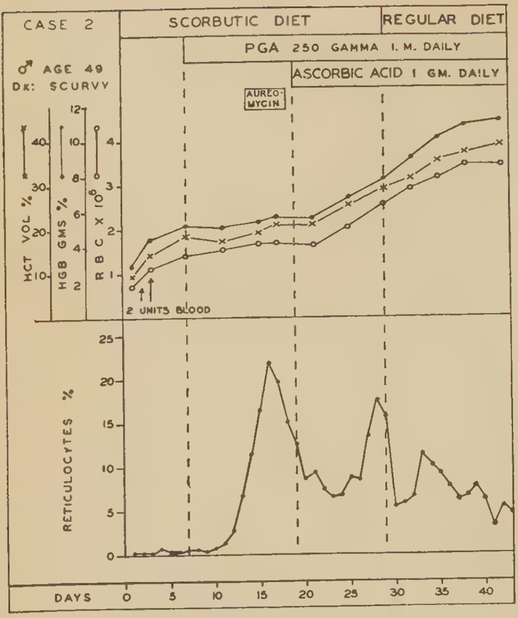

FIG. 2.

cyte responses were followed by increases in hemoglobin concentration, hematocrit, and red blood cell count.

In Case 2, the reticulocyte level was less than 1% during the initial control period (Fig. 2). On the tenth day of daily intramuscular PGA therapy a maximum reticulocyte level of 22% was noted. Three days after this response had begun a 4-day course of aureomycin (1 g p.o. daily) was given for an acute *E. coli* urinary tract infection. With administration of ascorbic acid a second reticulocyte rise occurred with a maximum value of 18% on the tenth day. During the period when the scorbutic diet was replaced by a regular diet with continuation of the PGA and ascorbic acid, a third reticulocyte response developed with a maximum value of 11% on the fifth day. The reticulocyte responses were associated with increases in hemoglobin concentration, hematocrit, and red blood cell count.

Urinary CF activity in Case 1 was immeasurably low throughout the study. In Case 2 urinary folic acid activity (FAA) increased somewhat during PGA administration (Table I). Urinary CF activity did not increase significantly after ascorbic acid therapy.

Discussion. The hematologic responses to the intramuscular administration of small doses of PGA observed in these patients suggest that PGA deficiency was involved in the pathogenesis of the anemia. Whether this PGA deficiency resulted from inadequate intake, from faulty utilization, or from both, cannot be stated with certainty. The secondary reticulocyte responses observed in both patients when ascorbic acid was given in addition to PGA may have resulted from a more efficient conversion of PGA to CF as the scorbutic state was corrected. This interpretation is supported by reports indicating that ascorbic acid facilitates the conversion of PGA to CF by rat liver slices(14), that CF is more effective than PGA in the anemia of scorbutic monkeys(20), and that patients with scurvy given large doses of PGA (10 mg p.o. daily) do not excrete normal quantities of CF in the urine until ascorbic acid is administered(18). The data on urinary excretion of PGA and CF obtained in the present study do not contribute to the interpretation of the hematologic data, since the increases in PGA excretion were small, and since no increase in CF excretion occurred following ascorbic acid administration. This may be related to the small doses of PGA used, to the retention of these substances for correction of tissue depletion, or to an increased requirement for them during active hematopoiesis.

The data presented here do not rule out the possibility that ascorbic acid *per se* might have been responsible for the secondary reticulocyte responses observed. However, previous experience in this laboratory has revealed no evidence of a direct stimulation of erythropoiesis by ascorbic acid alone in anemic scurvy patients. The present observations therefore indicate that ascorbic acid is necessary for optimal erythropoiesis in man through potentiation of the hematologic effect of PGA. This potentiation may be due to the more efficient conversion of PGA to CF which occurs when the scorbutic state is alleviated by ascorbic acid therapy.

Summary. Two men with scurvy and associated anemia, while maintained on a scorbutic diet, displayed hematologic responses to 125 and 250 μg of PGA, respectively, given intramuscularly daily. A potentiation of this

TABLE I. Urinary Folic Acid Activity (FAA) and Citrovorum Factor Activity (CF) in Case 2.

Days of study	Diet	Daily medication: PGA, μg i.m.	Daily medication: Ascorbic acid, g p.o.	Avg daily urinary excretion: FAA, μg	Avg daily urinary excretion: CF, μg
5– 7	Scorbutic	0	0	.505 (.330– .616)	.234 (.123– .323)
8–19	”	250	0	2.747 (.702– 6.280)	.506 (.156–1.036)
20–29	”	250	1	5.105 (1.360–13.524)	.502 (.332– .745)
30–43	Regular	250	1	5.739 (2.520–18.408)	.634 (.413–1.080)

hematologic effect of PGA occurred on subsequent administration of 1 g of ascorbic acid daily in addition to the PGA.

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Received October 19, 1953. P.S.E.B.M., 1953, v84.

Passage of Metabolites of Chloral Hydrate into Amniotic Fluid. (20675)

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The question of the origin of the amniotic fluid is still controversial. Gusserov(1,2) proved experimentally that fetal urine is excreted into the fluid, but according to Schroeder(3) the amniotic fluid is originally a transudate to which the fetal urine is added afterwards. Plentl and Hutchinson(4) estimated that in the human an hourly exchange of fluid amounting to 550 cc to and from the amniotic fluid takes place. The collective evidence suggests a possible change from a straight transudate at the time preceding fetal kidney function to a more or less exclusive renal origin in the later stage of fetal life. In our studies with chloral hydrate and its transfer from the maternal organism to the foetus, we found that the foetus excreted a metabolite of chloral hydrate in its urine and this was recovered in the amniotic fluid.

Butler(5) has shown that after chloral hydrate administration the blood contains, in addition to chloral hydrate, trichloroethanol and trichloroacetic acid. He also described a method of determination of these compounds in the blood, according to which chloral hydrate and trichloroacetic acid are directly determined colorimetrically by the red color developed by heating with pyridine and strong sodium hydroxide solution. Trichloroethanol is determined by the same method after oxidation of trichloroacetic acid with sulfuric acid and potassium dichromate. The urine of healthy animals contains after chloral hydrate administration only urochloralic acid as sole metabolite(6). This conjugation product of trichloroethanol with glucuronic acid cannot be determined directly, but after hydrolysis and oxidation with chromic acid mixture to form trichloroacetic acid, it can be determined as this compound by the method as described.

Methods. It is known that chloral hydrate readily passes the placenta and circulates in the foetus; however, no investigation has been extended to the amniotic fluid. Since human amniotic fluid is easily contaminated with blood, experiments were performed with rabbits. 1) Rabbit of 5160 g, in fourth week of gestation, received by stomach tube 200 mg chloral hydrate as a 10% solution. After 30 minutes the animal received a high dose of pentobarbital sodium intravenously, was immediately opened, and the amniotic fluid was withdrawn with a hypodermic syringe from the embryonic sacs. The clear liquid did not give the pyridine reaction, which excludes the presence of chloral hydrate or trichloroacetic acid. Extraction with heptane did not show the presence of trichloroethanol. After evaporation of 1 cc to dryness and oxidation with 2 cc chromate-sulfuric acid mixture, the reaction was positive, proving the presence of urochloralic acid. Colorimetric measurements indicated an equivalent to 4 μg chloral hydrate per cc. The analysis of the maternal blood showed per cc: chloral hydrate 10 μg; trichloroacetic acid 2.5 μg and trichloroethanol 2 μg. 2) Rabbit of 4430 g, gestation of 3 weeks, received 0.3 g chloral hydrate as 10% solution in oil rectally; after 1 hour 0.3 g in 10% aqueous solutions was given orally. Twenty-five minutes later the animal was killed. The amniotic fluid contained only urochloralic acid, 9.5 μg chloral hydrate equivalents per cc. The blood of this animal contained per cc: chloral hydrate 48 μg; trichloroacetic acid 12.5 μg and trichloroethanol 3.5 μg. 3) Rabbits, 5 kg weight, on 12th day of gestation. This is the earliest time at which pregnancy can safely be determined. The animal received 300 mg chloral hydrate as 10% solution in oil rectally, after one hour 100 mg as 10% aqueous solution orally, and was killed one-half hour later.

From 14 foetuses two specimens of pooled fluid were collected each from 7 foetuses. A blood sample was drawn before the killing and a urine sample was taken from the filled bladder. The amniotic fluid was obtained in small quantity and was of viscous consistency. Since the high protein content interfered with the color reaction, the liquid was treated with Folin's tungstic acid reagent and the tests carried out on the filtrate.

The analytical *results* were:

	Sample I	Sample II
Chloral hydrate	60 μg/cc	50 μg/cc
Trichloroacetic acid	22.5	15
Urochloralic acid (calculated as chloral hydrate)	44	35

Urine: Chloral hydrate and trichloroacetic acid negative; urochloralic acid: 560 μg/cc.

Blood: Trichloroethanol, 25 μg; trichloroacetic acid, 32.5 μg; chloral hydrate, 32.5 μg/cc.

In view of the small quantities of amniotic fluid collectable in the third rabbit, the potential error in the figures given is considered to be large, and the results should be evaluated rather as qualitative than quantitative.

It seems that at the time of gestation of the third rabbit, kidney function has set in and the transudative portion, containing the compounds circulating in the blood, is already mixed with urochloralic acid of urinary origin. At a later stage the urinary liquid replaces the reabsorbed transudate leading to a more watery fluid containing urochloralic acid only.

Summary. The results indicate that in the early stage of pregnancy chloral hydrate and its metabolites pass from the blood into the amniotic fluid. However, urochloralic acid, which is the form by which chloral hydrate is excreted by the kidneys, was found also at a stage when the gestation had progressed to two-fifths. In later pregnancy only urochloralic acid was found, which indicates that in the later stages the amniotic fluid contains essentially the products of foetal renal excretion.

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Received October 20, 1953. P.S.E.B.M., 1953, v84.

Blockade of Ovulation in the Hen with Adrenolytic and Parasympatholytic Drugs.* (20676)

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The involvement of the nervous system in the release of the ovulatory hormone (LH) has been demonstrated in several mammals. Electrical stimulation of the hypothalamus of the rabbit causes ovulation at levels that are ineffective when applied directly to the pituitary gland(1,2). Similarly other types of experiments on the rat have also indicated a neural component in ovulation(3). Recently Markee, Everett & Sawyer(4) have reviewed the data indicating the presence of a neural and neurohumoral link in the release of LH not only in the rabbit which ovulates following copulation but also in the rat, a non-spontaneous ovulator. The presence of a 24-hour rhythm in the neural link was indicated by the ability of progesterone to advance ovulation by 24 hours in the rat(5) and the ability of the barbiturates to delay ovulation by approximately 24 hours(6,7). Nembutal is also effective in preventing progesterone induced ovulation in rats(8).

The present study was undertaken to clarify the role of the nervous system in ovulation in the domestic hen. Like the rat, the hen is a cyclic, spontaneous ovulator. However, the hen differs from the rat in that the ovulatory

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* Aided by a grant from the Purdue Research Foundation.

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rhythm of the former is typically asynchronous with regard to the 24-hr day. The successive follicles of a clutch are ovulated progressively later each day until the clutch is ended by a terminal ovulation occurring around noon. In view of the findings in the rat it was deemed possible that a neurogenic factor is also present in ovulation in the hen. Recently we noted a failure on the part of Nembutal to block normal or progesterone-induced ovulation(9) indicating the possibility of a different mechanism in the bird. However, Fraps and Case (10) while confirming the above observations of Bastian and Zarrow(9) also reported premature ovulation with certain barbiturates. These authors suggested the possibility that the ovulation was due to neural excitation following administration of the barbiturates. Since the adrenolytic and parasympatholytic drugs have been used to great advantage in establishing neural control of ovulation in the rat and rabbit, the present study is concerned with the action of such drugs in the hen.

Material and method. Adult laying hens of the White Leghorn strain were used in this study. The birds were kept in a laying battery under conditions of constant temperature and humidity and supplied with food and water *ad libitum*. Artificial lighting was maintained from 8 A.M. to 9.P.M. daily. The time of ovulation was determined by digital palpation through the cloacca and also by recording the time of oviposition for all the birds by an automatic recorder(11). Records of the time of oviposition were kept for several weeks prior to each experiment in order to determine the characteristic, clutch length for each bird. All experiments involved the C_1 or first egg of the clutch and only hens laying a sequence of 2 or more eggs separated by a single day of failure to ovulate were used. Since LH release occurs 4 to 10 hours prior to ovulation (12,13) and ovulation of the C_1 follicle occurs from 4 A.M. to 7 A.M.(14), all injections were started at 8 P.M. preceding the day of expected ovulation. The atropine was dissolved in water at a concentration of 300 mg per ml and the adrenolytic drug, SKF-501,‡ was dissolved in a 50% mixture of propylene glycol and water at a concentration of 25 mg per ml.

TABLE I. Inhibition of Normal Ovulation in the Hen by Adrenolytic and Anticholinergic Agents.

No. of hens	Treatment	No. of hens ovulating	% blocked
12	Saline control	10	17
17	Atropine sulfate	5	71
27	SKF-501	6	79

Results. In the first experiment, each of 20 hens were injected hourly from 8 P.M. to 8 A.M. with 150 mg of atropine sulfate. The drug was given intraperitoneally in a 0.5 ml volume per injection and produced such symptoms as dropping of feathers, lowering of wings and panting. In some instances the birds appeared to be near death and the hourly injection was skipped. Three of the hens died and do not appear in the results. The SKF-501 was injected intramuscularly at a dosage of 10 mg per kg body weight. Each hen received an injection at 8 P.M. and a second at 1 A.M. No deaths were recorded for this treatment. Both of the drugs blocked the expected ovulation (Table I). Atropine produced a 71% block and SKF-501 a 79% block. Compared with the control birds that received only saline hourly for 12 hours, the inhibition by the 2 drugs is statistically significant.

The second experiment was designed to study the effect of the two drugs on progesterone-induced ovulation. One mg of progesterone was injected intramuscularly at 4 P.M. on the last day of the clutch *i.e.*, on the evening of the day preceding the next expected ovulation. The first injection of atropine was made at 3:45 P.M. *i.e.*, 15 minutes before the ovulation-inducing injection of progesterone and was continued hourly thereafter for 4 hours. The SKF-501 was injected intraperitoneally in a single dose of 10 mg per kg body weight at 3:45 P.M.

While progesterone alone caused 90% of the hens to ovulate, only 20% of the birds ovulated following treatment with atropine and progesterone and 56% of the birds ovulated following SKF-501 and progesterone (Table II). This represents an 80% block by atropine and a 44% block by SKF-501. In both instances the differences, when compared to

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‡ The authors are indebted to Dr. Fellows, from Smith, Kline & French, for the SKF-501.

TABLE II. Inhibition of Progesterone-Induced Ovulation in the Hen by Adrenolytic and Anticholinergic Agents.*

No. of hens	Treatment	No. of hens ovulating	% ovulating
10	Controls	9	90
10	Atropine sulfate	2	20
16	SKF-501	9	56

* All 3 groups of hens used in this experiment received 1 mg of progesterone intramuscularly.

the effect of progesterone alone, are statistically significant.

Discussion. The present results are consistent with the hypothesis established for the rat and the rabbit that a neural pathway is involved in the mechanism regulating ovulattion in the hen. Furthermore the action of atropine and SKF-501 would indicate that the neural pathway may have both an adrenergic and cholinergic link. The inhibition of both the normal and the progesterone induced ovulation by atropine and SKF-501 are identical with the results obtained in the rat(3) and the rabbit(15), and also indicate the presence of a neural factor in the mechanism that is concerned with ovulation in the hen. The present results however do not clarify the action of Nembutal with regard to its failure to prevent ovulation(9) and its ability to facilitate the process(10). Actual studies on the level of neural activity may be necessary before a definite explanation can be obtained. However, in spite of the failure of Nembutal to interfere with ovulation, these results indicate that a neural link is present in ovulation in the bird.

Summary and conclusions. Both atropine and SKF-501 block the normal occurring ovulation and the progesterone-induced ovulation in the hen. It is suggested that a neural link must be present in the mechanism regulating ovulation in the hen. This link may be similar to that postulated for the rat and the rabbit.

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Received October 22, 1953. P.S.E.B.M., 1953, v84.

Motility in a Species of Non-Flagellated Bacteria. (20677)

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Though there has been dispute concerning the role of flagella in bacterial motility, flagella have been found on most motile bacteria when examined under the electron microscope. Exceptions include members of the genera *Beggiatoa* and *Thiothrix* and of the order *Myxobacteriales,* in which creeping on a solid substrate has been described or in which flexible cell walls have been observed. In *Fusobacterium girans,* described in detail elsewhere (Macdonald)(1), motility which appears to be independent of flagella, creep-

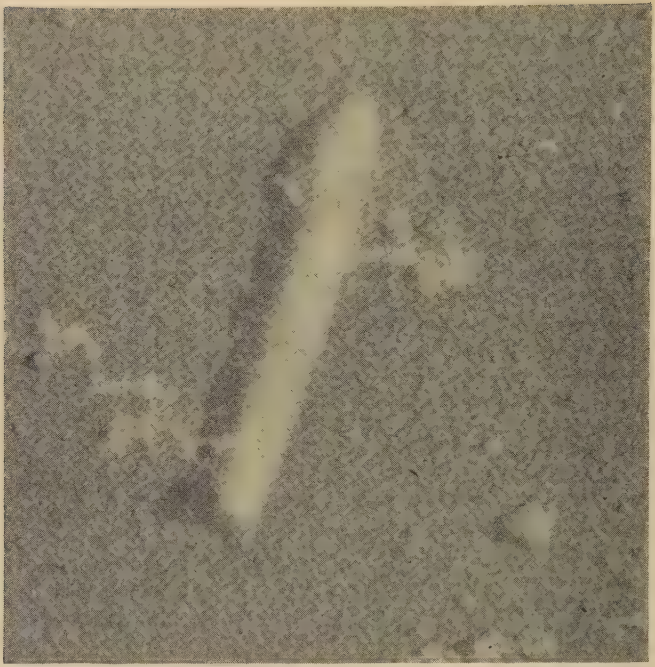

FIG. 1. Electron micrograph of strain WT-OZ, 3-day culture on blood agar. Shadow cast with chromium . × 24000.

ing, and flexibility, has been observed in a fluid environment.

Fusobacterium girans was originally described by Prevot(2) under the name *Fusocillus girans*. Five strains were described by Macdonald(1), who proposed the inclusion of these organisms in the genus *Fusobacterium*.* Examination of these same strains in respect to their motility forms the basis for the present report. All 5 strains appeared as tapered rods and filaments of various lengths. Motility was best seen from cultures in either sheep blood agar or ascitic fluid broth, both prepared with a veal heart infusion base. Organisms examined by darkfield microscopy exhibited 3 kinds of movement: (1) a sudden lashing around a fixed pole as if one end of the cell were fixed to the slide or coverslip, (2) flexing of the cell and (3) a gliding progression through the suspending medium at rates up to about 5 μ per second. The latter type of movement was seen in only relatively short cells (up to about 10 μ in length).

Darkfield examinations failed to disclose evidence of flagella in any of the 5 strains. No turbulence in excess of normal Brownian movement was seen in particles lying in close proximity to cells exhibiting progression. Nor was there evidence of rotation around the long axis accompanying this type of motility. It appeared as a simple migration either forward or backward and was only rarely associated with slight movements of flexion.

All 5 strains were examined under the electron microscope. Cultures were chosen in which most of the cells exhibited motility (including gliding progression in the case of 3 strains). Magnifications up to 24,000 were used and several hundred cells of each strain were examined. The resolution of the electron microscope under the conditions of these experiments was approximately 40A.

In no instance were flagella found in these examinations. The presence of a slime layer was suggested in some of the photomicrographs (*e.g.* Fig. 1). In preparations from the veal heart infusion broth containing an extract

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* The strains were reported to grow either anaerobically or in an environment of low oxygen tension, but not under ordinary aerobic conditions. Prolonged cultivation (more than two years) has resulted in growth on blood agar plates incubated aerobically. Motility has not been found in such cultures.

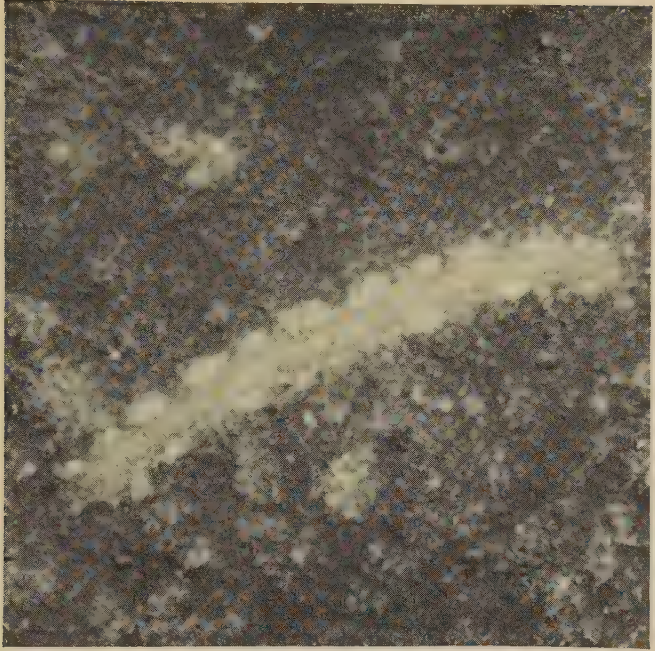

FIG. 2. Electron micrograph of strain WT-OZ, 3-day culture in broth. Shadow cast with chromium. × 12400.

of emulsified guinea pig kidney, particles frequently appeared adherent to the external surfaces of the cells as might occur if trapped in a slime layer (Fig. 2). Furthermore, India ink preparations counterstained with gentian violet disclosed ill-defined halos suggestive of a slime layer.

Examination of cultures by phase contrast microscopy and by polarized light failed to reveal any additional information. A slime layer was not visualized by these technics.

Lominski and Lendrum(3) reported the inhibition of motility in *B. proteus* and other organisms by the action of surface active agents. This effect was associated with loss of flagella attributed to either a selective lytic effect of the agents used or a failure of flagella to develop under conditions which did not favour motility. To test the effect of surface active agents on motility of *Fusobacterium girans*, 2 concentrations of each of 6 surface active agents were added to enriched veal heart infusion broth and the resultant media were inoculated with each of 5 strains of *Fusobacterium girans*. Four of the agents were anionic agents among those used by Lominski and Lendrum(3) and the dilutions used were in the range which was found by them to inhibit motility but not growth of several flagellated motile organisms (*i.e.*, sodium caprylate 1:500, 1:1000; sodium oleate 1:200, 1:400; sodium lauryl sulphate 1:2000, 1:4000; alkylated aryl sulphonate 1:2000, 1:14000). The remaining two were "Tween 20" (1:10, 1:100) and "Tween 80" (1:10, 1:100), non-ionic polyoxyethylene derivatives of sorbitan monolaurate and monooleate respectively.

In five days growth occurred only in the media containing alkylated aryl sulphonate (1:14000) and both concentrations (1:10 and 1:100) of "Tween 80". Motility was found in 4 of 5 strains in the medium containing "Tween 80" (1:10) compared to two of five strains in a control medium containing no added surface active agent. Motility in "Tween 80" (1:100) and in alkylated aryl sulphonate (1:14000) was found in only one of 5 strains. The experiment was repeated using only "Tween 80" and the control medium. The result was a finding of motility in 4 of 5 strains using "Tween 80" (1:10), 2 of 5 strains with "Tween 80" (1:100) and one of 5 strains in the control. The addition of "Tween 80" to the medium thus had no deleterious effect on motility and in fact may have enhanced it slightly. "Tween 80" (1:10) appeared to have no effect on motility of 12-hour cultures of *E. coli, S. paratyphi B, S. dysenteriae* and *Ser. marcescens.*

The influence of antiserum on motility was examined for 3 strains. Antisera prepared in rabbits for another purpose (Macdonald)(1), were used. Growth of each strain from a 3-day culture on blood agar was emulsified on a slide with homologous antiserum, covered with a coverslip and sealed with vaseline. Control emulsions in normal serum from the same rabbits were similarly prepared. All preparations were incubated at 37°C for 30 minutes and then examined by darkfield. The numbers of motile and non-motile cells in 10 fields were counted (a total of approximately 300 to 500 cells for each preparation).

In the control preparations motility was found in about 40% of cells of strain B8-3, about 5% of cells of strain WT-OZ, and about 2% of cells of strain MU-3. In emulsions prepared with antiserum, no motility was found.

Summary. Progressive gliding motility, lashing around a fixed pole, and flexion were found in five strains of *Fusobacterium girans* when examined in fluid suspensions by dark-field microscopy. Electron, phase contrast, and polarized light microscopy failed to reveal flagella on these organisms. The presence of a slime layer was indicated by electron microscopy, and by the use of India ink preparations. Motility apparently was not affected by the addition to the medium of either "Tween 80" or alkylated aryl sulphonate. Motility was inhibited in the presence of specific antiserum. No explanation is offered for the mechanism of motility in *Fusobacterium girans.*

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Received October 26, 1953. P.S.E.B.M., 1953, v84.

Hydrodextran: Preparation, and Study on Blood Level and Excretion Rate.
(20678)

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Native dextran is a long-chain glycosidic polymer of glucose produced by *Leuconostoc* fermentation of sucrose. For clinical use in blood-volume replacement, the native dextran is split by acid hydrolysis and then fractionated with methanol, to obtain shorter chain units in the range of 50-100,000 molecular weight. Each of these chains terminates in the hemiacetal group of the end-glucose unit. This reactive end-group is a possible source of destructive side reactions of dextran in solution, as pointed out by Zief and Stevens. These authors report the preparation of a hydrodextran in which the terminal hemiacetal groups are reduced to alcohol groups (4).

We have prepared a similar hydrodextran by means of high-pressure catalytic hydrogenation of clinical-fraction dextran. With the resultant modification in the terminal group of the polymer chain, it seemed possible that the mechanism of excretion from the blood stream might be different. For the blood level studies a 6% solution of hydrodextran, with 0.9% of sodium chloride, was prepared. In rabbits, and in man, it was

TABLE I.
Hydrogenation of Dextran, 12% Solution.

Analysis	Initial	Final
Viscosity, centistokes	6.65	6.22
Glucose equivalent, mg %	114	1.0

TABLE II. Stability of Hydrodextran, 6% Solution.

Specification	Initial	25°C	40°C	60°C
Hydrodextran, %	5.81	5.80	5.79	5.79
Molecular wt, wt avg	*	78000	81000	86000
Viscosity, centistokes	2.86	2.81	2.78	2.76
Glucose equiv., mg %	2.1	2.2	2.5	3.0
pH	6.7	6.5	6.5	6.5
Buffer capacity, ml 0.01N NaOH/l	0.5	0.8	0.8	0.7
Color, A.P.H.A.	15.	20.	20.	40.

* The molecular wt of the lower 5-10% of total material was 15000, that of upper 5-10% was 350000.

found that the blood level values progressively decreased in a pattern similar to that observed with dextran itself. No specific effect of the modified end-group was noted in these studies.

Preparation of hydrodextran. The initial dextran was prepared by acid hydrolysis of native dextran, followed by fractional precipitation with methanol to obtain material of 50-100,000 molecular weight. One hundred g of dextran was dissolved in 750 ml of water to give a 12% solution, and placed in an 1800-ml stainless steel rocking bomb together with 20 g of Raney nickel catalyst. The bomb was placed under 1000 lb pressure of hydrogen, and maintained at 140°C for 2 hours. The hydrogenated solution was filtered to remove the catalyst. As an indication of the course of reaction, the viscosity and glucose equivalent were run on both the initial and final solution. The glucose equivalent, as a measure of terminal glucose units, was determined by the Somogyi method(2). The hydrodextran was precipitated from the hydrogenated solution by the addition of 1.5 volumes of methanol. After standing overnight, the supernatant liquid was decanted and the solidified cake was dissolved by heating on the steam bath with 0.4 volume of water, based on the volume of hydrogenated solution. From this concentrate, the hydrodextran was reprecipitated as a fine, white powder by dropwise addition to 6 volumes of methanol, based on the volume used for the concentrate, with vigorous agitation. After partial settling, the supernatant liquid was decanted and replaced with fresh methanol. This slurry was filtered, the product was washed in the funnel with additional methanol, and air-dried. The final drying was for 20 hours in a vacuum oven at 10 mm and 110°C. The yield was 90% on a weight basis. In a solution of 6 g per 100 ml, the viscosity was 2.76 centistokes, and the glucose equivalent was 0.5 mg %.

Preparation of hydrodextran, 6% sol. For studies on administration of hydrodextran, the material was made up as a 6% solution with 0.9% of sodium chloride. The solution was filtered through a bacterial filter, drawn into 500-ml plasma-type bottles, and autoclaved for one hour at 250°F for final sterilization. A series of specification tests was run on this 6% solution, using the methods described for analysis of 6% dextran solution(3). For a measure of stability, 3 of the bottles were stored at different temperatures, and the specification values were redetermined after a period of 6 months. The data indicate a relatively stable preparation.

Excretion study in rabbits. For a preliminary study of the duration of plasma levels

TABLE III.
Duration of Plasma Hydrodextran in Rabbits.

Time	Plasma level, mg % 1	2	3
Control	3.5	3.5	4.5
Immediate	1332	1672	1540
24 hr	285	339	346
48 "	84	115	76

TABLE IV.
Duration and Excretion of Hydrodextran in Man.

Time	Plasma level, mg % 1	2	Total in urine, g 1	2
Control	21	43	—	—
Immediate	723	723	—	—
6 hr	284	303	16	17
12 "	260	250	17	18
24 "	202	190	18	18
48 "	133	108	18	18

of hydrodextran following intravenous administration, three 1.75-kg male rabbits were given 30 ml each of the 6% solution. The hydrodextran content of the plasma was determined by colorimetric assay of the alcohol precipitate, after digestion of the plasma sample with potassium hydroxide(1).

Excretion study in man. Hydrodextran in 6% solution was administered intravenously to 2 subjects. Samples were taken to determine the plasma hydrodextran concentration. The total urine was collected and analyzed to determine the cumulative excretion of hydrodextran. The samples were analyzed by the alcohol precipitation method referred to(1). The amount given was 500 ml of a 6% solution, or 30 g of hydrodextran, injected over a 30-minute period. The picture observed was essentially the same as that reported for dextran itself(1).

Summary. Hydrodextran was prepared by high-pressure catalytic hydrogenation of dextran. The 6% solution prepared for intravenous use was found to be essentially stable over a 6-month period of storage. The preliminary studies on blood level and urine excretion showed no specific effect from hydrogenation of the terminal groups of the dextran chain.

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Received January 16, 1953. P.S.E.B.M., 1953, v84.

Growth Promotion in Rats by Crude Concentrates of the Bifidus Factor.*

(20679)

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A growth-factor essential for a distinct strain of *L. bifidus* var. Penn is present in human milk and in particular in human colostrum. In contrast, the activity of cow's milk with regard to the same microbiological growth-factor is very low, on the average only 1/40th that contained in human milk(1-3). This "Bifidus Factor" occurs in low and high molecular form and appears to be related chemically to the blood group polysaccharides (1-3). Hog mucin and meconium, as well as other sources of blood group polysaccharide, have shown high microbiological activity for *L. bifidus* var. Penn(1-3). The recognition of a specific, microbiologically active factor in human milk made it desirable to investigate its possible role in the nutrition of animals and men. In the present study, supplements of crude sources of the Bifidus Factor were added to special rations fed young rats and their effect on growth and utilization of food was observed.

Material and methods. In the first and largest group of experiments a commercial infant formula was used,‡ which in its composition, at least in its content of total protein, fat and lactose, closely approximates that of human milk. The addition of concentrates of the Bifidus Factor should make this mixture even more similar to human milk. This dilute cow's milk formula was fed to young weanling rats of the Sprague Dawley strain *ad libitum.* Quantity consumed and weight of rats was measured daily. Control animals received the unsupplemented formula. The various supplements used in the experiments were as follows:

Exp. 1. Duration 8 weeks a) Charcoal eluate from human milk(4) with average microbiological activity of 300 γ per unit, 400 mg

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* Supported by a grant from Wyeth Laboratories, Division of American Home Products Corp.

† On a Fellowship from the Oswaldo Cruz Institute, Rio De Janeiro, Brazil.

‡ S. M. A. liquid, Wyeth Laboratories. For use, contents of one can (13 oz.) was diluted with water to make 1 liter.

per 100 ml basal diet, b) Meconium-suspension (20%): 15 ml, equivalent to about 1000 microbiological units of Bifidus Factor, in 100 ml of basal diet, c) Hog mucin, granular (Wilson), microbiological activity 300 γ per unit, daily dose 50 mg per rat. *Exp. 2.* Duration 10 weeks a) Hog mucin, 600 mg per 100 ml of the basal diet, b) Human colostrum, with average microbiological activity 5 times higher than late human milk(2,3): 20 ml in 100 ml basal diet, c) Meconium-suspension (20%): 15 ml in 100 ml basal diet, d) "Activated" chitin(5). Chitin, prepared from crabshells, was treated with HCl and methanol(5), microbiological activity about 300 γ per unit, given in amount of 300 mg per 100 ml basal diet. *Exp. 3.* Duration 4 weeks a) Hog mucin, 400 mg per 100 ml of the basal diet, b) Hog mucin, hydrolyzed with HCl, microbiologically inactivated,§ given in equivalent amounts as unhydrolyzed hog mucin, c) Hog mucin treated with an enzyme preparation, obtained from *L. bifidus* var. Penn(1-3), microbiological activity reduced to about 20-30% of the original activity, given in equivalent doses as untreated mucin, d) Hog mucin mixed with inactive (boiled) "Bifidus enzyme", in the same amount as used for inactivation of hog mucin with active enzyme. In these last 2 preparations, the lyophilized enzyme preparation containing about 25% protein was added to an equal amount of hog mucin. Measured weight gains were compared with total protein intake over the entire period of the experiments, and the data were calculated according to customary statistical methods. The *basal diet* was considered to contain 70 cal/100 ml and 1.5 g protein/100 ml. 20% human colostrum in the basal diet should give values of 68 cal/100 ml and 1.8 g protein/100 ml(6). The milk eluate was free from protein. Mucin contained about 50% of protein. No similar calculations were required for meconium and "activated" chitin, which were found to have no growth promoting effect. In Exp. 4, the same dilute milk-formula was used as in Exp. 1-3 for a period of 8 weeks. The formula was supplemented with most of the known vitamins, *i.e.*, A, D, E, K and those of

TABLE I. Effect of Bifidus Factor on Growth and Food Efficiency in Rats Fed Cow's Milk Formula.

Exp.	Wk		Animals	Wt gain, g	Prot. intake, g	Cal. intake (hundreds)	Gain/Prot.	P*	Gain/Cal. (100)	P
I	8	Control	10	28.2 ± 2.5†	24.0 ± .3	11.2 ± .1	1.2 ± .1		2.6 ± .2	
		Milk eluate	8	42.8 ± 2.0	24.0 ± .3	11.2 ± .1	1.8 ± .1	.001	3.9 ± .2	.005
		Meconium	9	26.7 ± 1.5	23.0 ± .3	10.7 ± .1	1.2 ± .1		2.6 ± .2	
		Hog mucin, 50 mg/day	9	38.0 ± 2.3	25.0 ± .2	11.7 ± .1	1.6 ± .1	.001	3.4 ± .2	.005
II	10	Control	9	44 ± 2.8	31 ± .7	14.4 ± .32	1.4 ± .04		3.1 ± .09	
		Hog mucin, 600 mg/100 ml	12	82 ± 3.4	48 ± .8	19.2 ± .32	1.7 ± .09	.01	4.3 ± .23	.001
		Colostrum, 20%	11	83.8 ± 3.5	48 ± .9	18.1 ± .36	1.7 ± .09	.01	4.6 ± .25	.001
		Chitin ("activated"), 300 mg/100 ml	12	48 ± 2.7	32 ± .8	14.9 ± .32	1.5 ± .06	.3	3.2 ± .13	
		Meconium, 15 ml 20%/100 ml	12	39.8 ± 3.2	32 ± .8	14.9 ± .32	1.2 ± .09		2.7 ± .20	
III	10	Control	10	16.4 ± 3.0	13.5 ± 1.0	6.3 ± .5	1.2 ± .12		2.5 ± .3	
		Hog mucin, .4%	10	42.0 ± 3.7	21.8 ± 1.7	9.2 ± .4	1.9 ± .10	.001	4.5 ± .2	.001
		Hydrol. hog mucin, .4%	10	17.0 ± 2.1	15.4 ± .9	6.5 ± .4	1.1 ± .12		2.5 ± .3	
		Mucin + enzyme, .4%	10	38.2 ± 3.8	21.8 ± 1.2	8.7 ± .5	1.8 ± .09	.001	4.3 ± .2	.001
		Mucin + boiled enzyme, .4%	9	33.0 ± 4.7	21.0 ± 1.5	8.4 ± .6	1.6 ± .15	.05	3.8 ± .3	.01

* P = Probability calculated from distribution of *t*, Fisher, R. A., and Yates, F., Statistical Tables, London, 1938, p. 26.

† All values following ± are stand. error of the mean: $\frac{\text{Stand. dev.}}{\sqrt{\text{No. observations}}}$.

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§ Kindly supplied by Dr. R. M. Tomarelli, Wyeth Laboratories, Mason, Mich.

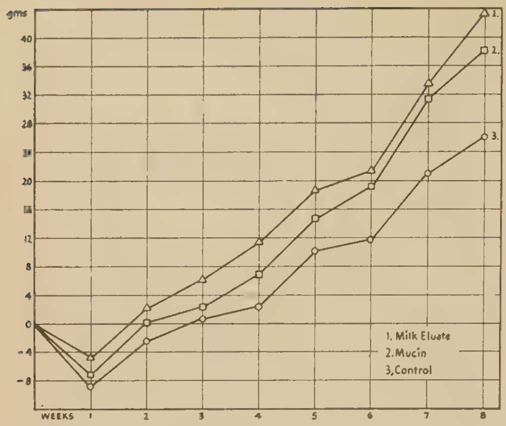

FIG. 1. Weight curves of rats receiving milk eluate and mucin compared with controls.

the vit. B Complex, including folic acid (10 γ daily), biotin (1 γ) and B_{12} (0.1 γ). The control group received 0.4% glucose added to the milk dilution, 4 experimental groups were supplemented with 0.4% of hog mucin, glucuronolactone,[∥] galacturonic acid and pectin respectively.

In a second series of experiments, the basal diet consisted of 30% alcohol-extracted peanut meal, 6% vitamin-free casein, 40% sucrose, 20% lard and 4% salt mixture, with the usual vitamin supplements, except choline(7). This and similar rations were used for the production of fatty liver and hepatic cirrhosis. In this paper, only the growth-promoting effect of hog mucin is reported (Exp. 5). The results obtained in this experiment were confirmed in other similar experiments.

Results. The experimental observations (Exp. 1-3) made on rats fed dilutions of cow's milk as basal diet with or without supplement are summarized in Table I. The weight curves from Exp. 1 are charted in Fig. 1. The addition of mucin, of charcoal eluates prepared from human milk and of human colostrum have proved to have growth promoting effect. They also improved the utilization of ingested calories and of protein consumed, and thus of food efficiency. In contrast, meconium and "activated chitin", consisting chiefly of α- and β-methylglucosaminide(5) were found to be inactive. Acid hydrolysis of mucin deprived it of its growth promoting activity, whereas enzymatic inactivation up to 70-80% left the nutritional benefit exerted by mucin unimpaired.

The findings in Exp. 4 may be summarized as follows: *Exp. 4.* Average starting weight of rats in all 5 sub-groups 54.0 g. Weight-gain after 8 weeks in the experiment with glucose 41.5 ± 2.0, mucin 67.2 ± 3.3, glucuronalactone 41.8 ± 2.4, galacturonic acid 47.8 ± 3.1 and pectin 47.3 ± 3.1. The greater increase in weight in the group receiving mucin compared with all other control groups is highly significant ($P < 0.001$).

Even with the use of a high-protein alipotropic diet, consisting chiefly of peanut-meal, the growth promoting effect of hog mucin was clearly demonstrable over a prolonged experimental period (Table II).

Discussion. Human colostrum, eluates from human milk, and hog mucin, which all promote growth in rats under special experimental conditions, are characterized by high microbiological growth activity for *L. bifidus* var. Penn. On the other hand, "activated chitin" and meconium, both microbiologically active, were in comparable doses ineffective in animals. There are different possible explanations for this discrepancy:

1. Microbiological activity may be less specific and could be inherent to a varying number of chemically similar compounds, acting perhaps as precursors for the real growth-factor. In contrast, growth effect in animals is exerted only by compounds containing the "true" Bifidus Factor. Thus, meconium and "activated chitin" would not belong in this category.

2. Only high molecular forms of the Bifidus Factor(1-3) may influence, beneficially, growth in animals, probably through the intermediary of the intestinal flora. "Activated chitin" consists chiefly of low molecular compounds. Meconium may be ineffective, because its high molecular, microbiologically active constituents are unavailable to the intestinal flora of rats.

The growth effect in rats by colostrum, eluates from human milk, and hog mucin may not be due to the Bifidus Factor but to other, unidentified "contaminants". The fact that acid-hydrolyzed mucin was inactive would

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[∥] Kindly furnished by Corn Products, Inc.

TABLE II. Effect of Hog Mucin on Weight-Gain in Rats Fed an Alipotropic Peanut Meal Ration for 200 Days.

Exp.	Days		Animals	Starting wt, g	Final wt, g	P	Food intake ag. daily, g
V	200	Control	10	115.0 ± 3.0	292 ± 12.5		10.6 ± 0.3
		Hog mucin, 25 mg/day	10	115.7 ± 1.9	323 ± 6.0	.05	10.7 ± 0.4
		50 mg/day	9	115.4 ± 1.8	330 ± 8.1	.02	10.7 ± 0.4

rather support the determining role of the Bifidus Factor. On the other hand, the unimpaired activity of enzymatically treated hog mucin with greatly reduced microbiological activity does not favor, although it does not exclude this conclusion. It is conceivable that some special high molecular compounds of hog mucin, not inactivated by the enzymatic process, are the growth promoting agents for rats. Rearrangement through transglucosidation in the intestine is equally possible.

The results with hydrolyzed mucin, as well as those with small amount of mucin added to a high protein (peanut-meal) diet, and finally negative experiments(8) with substitution of mucin by equivalent quantity of protein (casein) seem to eliminate the possibility that the effect of crude mucin in animals may be due to its protein content. Furthermore, the active eluates obtained from human milk were free from protein and contained mainly bifidus-active polysaccharides.

The growth rate of the experimental animals, even when fed active concentrates, has been subnormal, due to the low total protein content of the basal dilute milk ration. Addition of vitamins, or of glucose, glucuronolactone, galacturonic acid, or pectin to the basal diet was without any effect on the growth rate, whereas hog mucin, polysaccharide fractions of human milk or colostrum have shown distinct growth promoting effect. Glucuronolactone(9-11) and other carbohydrates, such as chondroitin sulfuric acid(12-14) have been in the past credited to act under particular experimental conditions as growth promoting factors for chicks and rats. Chondroitinsulfuric acid is present in hog mucin(15). On the other hand, the fractions obtained from human milk are free from sulfur. Thus, at least for the fractions prepared from human milk, chondroitin sulfuric acid is not the growth promoting constituent.

Summary. Human colostrum, charcoal eluates prepared from human milk, and hog mucin, promoted growth in rats receiving a dilute cow's milk formula as basal diet. Supplements of known vitamins or of glucose, glucuronolactone, galacturonic acid, and pectin were ineffective. Rats receiving an alipotropic ration, containing peanut-meal and casein as source of protein, have shown better weight gain after supplementation with small daily doses of hog mucin than the unsupplemented controls. The question was raised and discussed whether these beneficial effects may be related to the Bifidus Factor activity of the supplements used.

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Received July 8, 1953. P.S.E.B.M., 1953, v84.

Vitamin E Deficiency and Xanthine Oxidase in Rabbits.* (20680)

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Dinning has reported(1) that vit. E-deficient rabbits have an unusually high concentration of xanthine oxidase (XO) in the liver. This has been confirmed in the present study, and it has also been found that this increase occurs only in the liver and is not accompanied by any parallel change in the molybdenum concentration. The latter point is of interest because of the recently demonstrated(2) relationship between molybdenum and xanthine oxidase.

Experimental. White male New Zealand rabbits, weighing approximately one kg, were separated into groups and fed various diets until muscular dystrophy appeared in the vit. E-deficient animals(3). The deficient and control rabbits were then sacrificed and analyzed. In the first experiment a large number of rabbit tissues were analyzed for xanthine oxidase in the presence and absence of methylene blue by the methods previously described(4). In the second experiment, only the liver was analyzed for xanthine oxidase in the presence of methylene blue, since the methylene blue procedure gave the most reliable results; in addition, a large number of tissues were dried extensively at 80°, digested with sulfuric-nitric-perchloric acids, and analyzed for molybdenum colorimetrically(5,6).

The 15% casein diet described by Young and Dinning(7), and also used by Dinning(1) in his studies on liver xanthine oxidase during vit. E-deficiency, was used. Both experiments employed this diet with and without the addition of 50 mg of mixed tocopherols per 100 g diet. In the second experiment 2 additional diets containing 2 mg Mo (as Na_2MoO_4) per kg diet were used, and a third vit. E-Mo-deficient diet, containing 24% casein and 30% sucrose, was also studied. Purina rabbit chow, containing 15% protein and 1.84 mg Mo per kg, was fed to another control group of rabbits in each experiment.

Results. In the normal chow-fed rabbits no xanthine oxidase could be detected in the presence or absence of methylene blue in heart, lung, kidney, spleen, brain or skeletal muscle. Nor could xanthine oxidase be detected in these tissues during vit. E-deficiency in those rabbits which showed a marked elevation in lixer XO. Liver and small intestine from normaly chow-fed rabbits contained small amounts of this enzyme, which could be detected in the presence of methylene blue, but not regularly in its absence.

The results obtained with liver and intestinal xanthine oxidases in the first experiment are shown in Table I. Although there was considerable variation in the magnitude of the increase in liver XO during vit. E-deficiency, there was no question of its occurrence; 4 of the 7 deficient rabbits had liver XO values (as determined in the presence of methylene blue), between 20 and 50, and these far exceeded any of the control values. The diets had little or no effect on the low intestinal xanthine oxidase levels.

The effect of vit. E-deficiency on the endogenous respiration of a rabbit liver homogenate is shown in Fig. 1. These curves are reminiscent of those previously published(8) for rat liver, and show that the endogenous oxygen uptake was low when the liver contained little XO (controls); the endogenous respiration increased with the elevated liver xanthine oxidase found in vit. E-deficiency. The effect of methylene blue on the determination of xanthine oxidase in both normal and vit. E-deficient rabbit liver was similar to its effect in a variety of normal tissues (8-12), and was apparently unrelated to any effect that methylene blue might have on vit. E deficiency.

Table II shows the results of the second experiment. Liver xanthine oxidase was again elevated on all vit. E-deficient diets; the pres-

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* This study was aided by a grant from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

TABLE I. Effect of Vitamin E Deficiency on Liver and Intestinal Xanthine Oxidase Activity in the Rabbit.

	Days on diet	Body wt, g		No. of rabbits	Liver XO*		Intestine XO*	
		Start	End		—	+MB	—	+MB
Chow	39	1130	2045	3	2 ± 1.0	5 ± 2.5	0	4 ± 0.7
Purified diet with vit. E	32	1086	1086	5	0	7 ± 2.5	0	9 ± 3.0
Purified diet without vit. E	29	1001	851	7	12 ± 6.0	26 ± 6.9	0	7 ± 2.9

* Liver and intestine xanthine oxidase activities were determined in the presence and absence of methylene blue, and have been recorded as net mm³ O_2 per 20 min. per flask containing 283 mg fresh tissue; mean ± stand. error.

TABLE II. Effect of Dietary Vitamin E and Molybdenum on Xanthine Oxidase and Molybdenum Concentrations in Rabbit Tissues.

Diet			Body wt, g				Mo content—γ/g dry wt							
Vit. E	Mo	Avg days fed	Start	End	No. of rabbits	Liver xanthine oxidase†	Kidney	Liver	Intestine	Stomach	Colon	Lung	Skin	Muscle
—	—	33	1161	979	6	9 ± 1.9	1.3	.9	.6	.6	.5	.7	.2	.2
+	—	37	1099	1265	5	1 ± 0.5	1.7	.7	—	—	.5	.8	.3	.2
—	+	28	1154	1012	6	17 ± 6.0	2.6	1.4	1.5	1.2	1.1	1.4	.6	.3
+	+	43	1089	1228	6	3 ± 2.8	3.6	1.6	1.6	1.1	1.2	1.5	.6	.4
—	—*	28	1095	1051	5	23 ± 6.0	1.5	1.0	.7	.4	.6	.8	.4	.2
Chow		40	1134	2470	6	5 ± 2.5	2.0	1.5	1.1	.8	.8	.6	.3	.2

* 24% casein diet; all others contained 15% casein; chow contained 15% protein.

† Xanthine oxidase activities were determined in the presence of methylene blue, and have been recorded as net mm³ O_2 consumed per 20 min. per flask containing 283 mg fresh tissue; mean ± stand. error.

ence or absence of molydenum and the use of 24% instead of 15% casein in the diets did not prevent this increase. Nor did the addition of Mo to the diet influence the control level of liver xanthine oxidase or the appearance of the muscular dystrophy deficiency symptoms. Comparison of the magnitude of the increase obtained with different diets was not justified, because the change in liver XO has been reported(1) to occur late in the deficiency and the variability among rabbits within the deficient groups might be influenced by the severity of the deficiency at the time of sacrifice. It is possible that the purified diet contained enough molybdenum, as an impurity, to meet the requirements of the rabbit.

The molybdenum content of the rabbit tissues reflected the Mo content of the diet, and was not affected by the presence or absence of vit. E or by the difference in protein content. Vit. E deficiency did not alter the molybdenum content of the liver or the distribution of Mo in the other tissues, and there was no correlation between the xanthine oxi-

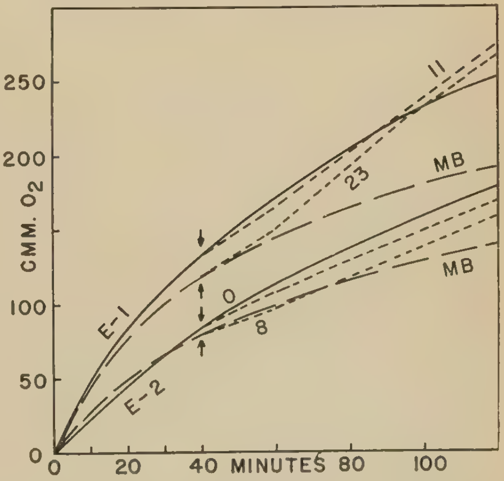

FIG. 1. The avg endogenous respiration (solid line) of liver homogenates from 6 vit. E-deficient (E-1) and 5 control (E-2) rabbits. Dash lines were obtained in presence of methylene blue (MB), dotted lines show effect of tipping in hypoxanthine substrate at the 40 min. point. Numerical values along the dotted lines are corresponding xanthine oxidase activities in net mm³ O_2 per 20 min. per flask containing 283 mg fresh tissue.

dase activity of the liver and its molybdenum concentration.

Summary. Vit. E-deficient rabbits with muscular dystrophy had elevated liver xanthine oxidase activity. This increase was observed in the presence or absence of added dietary molybdenum, and with 15 or 24% casein in the diet. Low intestinal xanthine oxidase levels were not increased during vit. E deficiency. Xanthine oxidase was not detected in rabbit heart, lung, kidney, spleen, brain, or skeletal muscle normally or during vit. E deficiency. The molybdenum content of various rabbit tissues was influenced by the Mo content of the diet, but was not altered by the presence or absence of vit. E.

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Received July 13, 1953. P.S.E.B.M., 1953, v84.

Effect of Whole Body X-Irradiation on Ascorbic Acid of Rat Tissues.* (20681)

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Interest in the fate of ascorbic acid in irradiated tissues has been stimulated by several reports. It has been shown(1) that X-irradiation of ascorbic acid in aqueous solutions results in a loss of from 1.7 to 2.4 μ moles/1000 r. This is the order of magnitude of the effect of X-irradiation on a number of organic compounds in dilute solution(2). The destruction of ascorbic acid by X-irradiation, unlike thiamine and most organic compounds, is not inhibited by the presence of albumin and, indeed, proceeds to the same extent in blood plasma as in aqueous solution. However, only slight destruction of ascorbic acid occurred in minced rat muscle exposed to 22,000 r *in vitro* (1). Favorable clinical reports on the use of ascorbic acid in the treatment of X-ray sickness led to the study of the effect of X-ray therapy on the serum ascorbic acid of patients by Kretzschmar and Ellis(3). A reduction was noted during and after treatment. These workers also reported an immediate and sustained (12-30 days) reduction in plasma ascorbic acid of rabbits which had received 1500 r on one side of the body. Moreover, they found a reduction in muscle and kidney ascorbic acid in rats 1-48 hours after irradiation of these tissues. These reports indicate the possibility that cell ascorbic acid might be destroyed by the production of oxidizing radicals in cell water during X-irradiation(4). This type of immediate *in vivo* destruction has been reported by Skoog(5) to occur in the auxin of plants receiving from 600-2100 r. The prolonged effect on plasma ascorbic acid found by Kretzschmar and Ellis suggests that besides the possible primary oxidative destruction, there are also secondary factors which influence the *in vivo* ascorbic acid levels after

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* This study carried out under Contract for U.S.A.E.C. at University of Michigan.

X-irradiation. One of these factors might be reduction in food intake. In species not dependent upon dietary intake for ascorbic acid this factor should not be significant in relatively short term experiments. The report of Patt *et al.*(6) on adrenal weight and cholesterol content after whole body irradiation has established that irradiation activates the pituitary-adrenal system. Pituitary-adrenal activation might, therefore, be expected to be another factor in the alteration of *in vivo* ascorbic acid levels. Wexler *et al.*(7) have recently reported changes in adrenal ascorbic acid, after whole body irradiation, attributable to this factor.

Our investigation was undertaken to extend the earlier work to whole body irradiation and to other tissues. Data will be presented which indicate that some oxidation of ascorbic acid may occur in irradiated rats of the Long-Evans strain. In rats of the Wistar strain, on the other hand, the principal changes are the result of activation of the pituitary-adrenal system.

Procedures. Nineteen female Long-Evans (Rockland Farms) rats, body weight 138-208 g, and 54 Wistar (Carworth Farms) rats, body weight 94-162 g, were used. They were given tap water and Rockland Rat Diet (complete) fed *ad libitum.* This diet was supplemented with fresh leaf lettuce twice weekly. During the 72 hour post-irradiation period of study, very little food was eaten by the irradiated animals.

Exp. 1. Ascorbic acid was determined by the method of Roe and Kuether(8) in the adrenal gland, thymus, spleen, liver, kidney, thigh muscle, and whole blood. The animals were anesthetized with ether and were sacrificed by bleeding from the inferior vena cava. The blood was drawn into the required(9) amount of heparin (7.35 mg/ml) in saline solution. The tissues were then removed, dissected free of fat, weighed and placed into a pyrex homogenizer(10) with the required amount of 4% trichloroacetic acid (TCA), and carefully homogenized for one minute. Six liver homogenates made in this way contained an average of 15.8 mg ascorbic acid/100 g liver. When the sediment, from a centrifuged homogenate, was rehomogenized and the 2 supernatant layers combined, the average ascorbic acid value was 17. The difference is within experimental error, indicating that there was no additional ascorbic acid obtained by rehomogenization. Furthermore, hydrolysis of the homogenate by 1 N acetic acid (steam cone for 45 minutes, in an H_2S atmosphere) yielded no increment. It was therefore concluded that a single extraction was sufficient for the complete removal of ascorbic acid.

TABLE I. Tissue Ascorbic Acid Values for Control Animals of Wistar (W) and Long-Evans (L-E) Strains. All values are expressed as mg per 100 g (tissue) or ml (blood).

Organ	Mean ascorbic acid value	
	L-E	W
Adrenal	330 ±30. *	380 ±20. *
Thymus	39 ± 3.	33 ± 1.
Spleen	33 ± 3.	38 ± 1.
Liver	19 ± 2.	19 ± 1.
Kidney	8.9 ± .4	8.2 ± .5
Muscle	3.2 ± .3	2.2 ± .1
Blood	.81 ± .09	.58 ± .04

* Stand. error of mean.

TABLE II. Effect of Irradiation on Tissue Ascorbic Acid of Long-Evans Strain Rats. (All values expressed as mg per 100 g (tissue) or ml (blood)).

Organ	Difference from control immediately after irradiation
Adrenal	—70 ±36 *
Thymus	— 4.0 ± 4.2
Spleen	— 5.0 ± 3.6
Liver	— 2.0 ± 2.8
Kidney	+ .4 ± .8
Muscle	— .9 ± .4
Blood	— .24 ± .11

* Stand. error of difference $= \sqrt{(\sigma m_1)^2 + (\sigma m_2)^2}$.

Ten unirradiated rats of the Long-Evans strain were sacrificed and the ascorbic acid of the tissues analyzed as indicated. These data constituted the *control values* (Table I). Subsequently 9 animals received 710 r whole body X-irradiation, and the same analyses were made immediately after irradiation. The irradiation factors were: 200 KV, 20-23 MA, ½ mm Cu + 1 mm Al filters, 19.3-20.0 r.p.m., and 86 cm t.o.d.

Exp. 2. A total of 54 female Wistar rats were used. The procedure was the same as in Exp. 1 except that a control animal was sacrificed with each irradiated animal. Ten irradiated animals, with controls, were sacrificed immediately after irradiation. Of these, 4 animals received 710 r, three 800 r, and three

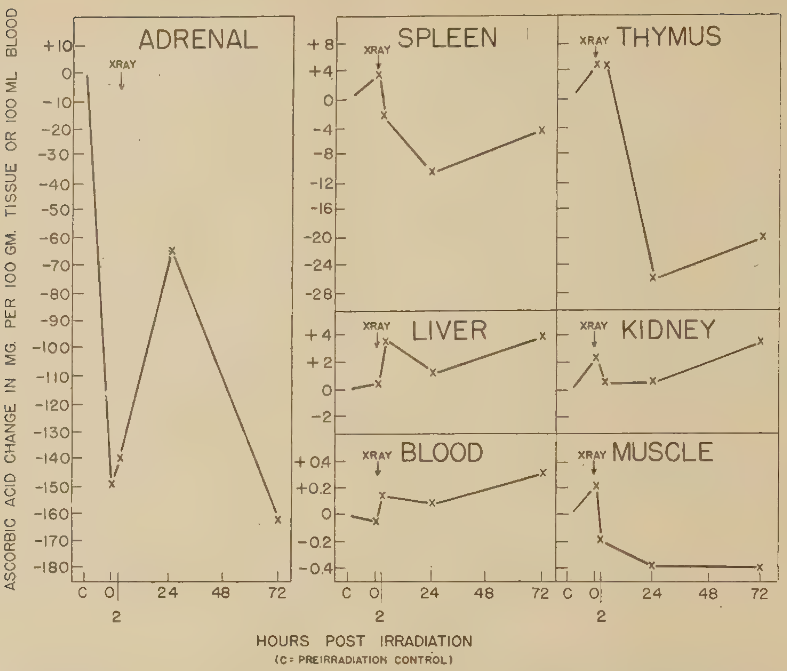

FIG. 1. Changes in tissue ascorbic acid of Wistar female rats after 1000 r whole body x-irradiation. Units of ordinates are chosen to represent one standard deviation of differences plotted, except in the case of the adrenal where each unit represents a quarter of a standard deviation.

1000 r. Since there was no marked difference attributable to the change in dose, these animals are considered together and all subsequent groups received 1000 r. Two groups, each of 5 irradiated animals with 5 controls, were sacrificed 2 and 24 hours after irradiation, and 7 irradiated animals with 7 controls were sacrificed 72 hours after irradiation.

The data of Exp. 1 are presented in Table II, and of Exp. 2 in Fig. 1. In each case the difference between irradiated and control animals is presented. (K. Mather, quoted in (3)).

Discussion. From Table II it can be seen that in Long-Evans rats irradiated at 710 r (approximately a mid-lethal dose) a statistically significant decrease in the ascorbic acid content of the adrenals, muscle, and blood occurs immediately after irradiation. Less marked decreases occurred in the thymus, spleen, and liver. Our data for the Long-Evans rat, therefore, confirm the findings of Kretzschmar and Ellis(3), that the ascorbic acid of muscle and plasma is decreased immediately after irradiation, and show a marked decrease in adrenal ascorbic acid as well. That this immediate decrease in muscle and blood ascorbic acid is dependent upon the strain of the animal studied is suggested by the results of Exp. 2. The strain dependence of response to irradiation has been repeatedly emphasized.

The data of Exp. 2 for the animals sacrificed immediately after irradiation are plotted (Fig. 1) as zero hour points although it required about 50 minutes to deliver the dose. The earliest changes, in tissues other than the adrenal, as brought out in Fig. 1, are not individually statistically significant. However, consideration of the 4 tissues—thymus, spleen, liver, and kidney—together suggests a tendency for the earliest values in the irradiated animals to be higher than the controls. It is possible that this represents a synthesis of vitamin by injured cells as reported by Loofbourow and his associates for various living tissues (rat, mouse, and chicken embryos and adult newt), subjected to lethal ultraviolet radiation, X-irradiation, mechanical and chemical injury(11-13). Of interest also, is the report of Richmond, Altman and Salomon

(14), that bone marrow and spleen homogenates from animals sacrificed immediately after irradiation exhibit an increased capacity for the synthesis of hemin and globin.

The changes presented in Fig. 1, are probably due to activation of the pituitary-adrenal system as a result of the "non-specific stress effect" of X-irradiation(6,7,15). It is particularly to be noted that the changes in the liver and blood are the opposite of the change noted in the adrenals. Sayers *et al.*(16) have reported a rise in liver and plasma ascorbic acid with reduction of adrenal ascorbic acid caused by loss of 1.1 ml blood per 100 cm^2 of body surface. The finding of an adrenal ascorbic acid level below normal at 24 hours and a secondary more severe reduction at 72 hours, we attribute to the severity of the irradiation which is at least 100 r above the dose required for about 90% mortality. A biphasic change of this type in the adrenal cholesterol has been reported(6) in rats receiving 900 r whole body irradiation.

The decrease in ascorbic acid of the muscle, thymus and spleen in Exp. 2, since it occurs after an initial increase, is probably not due to direct oxidation of the vitamin. The data indicate that the changes in ascorbic acid which occur in the tissues of rats of the Wistar strain are due to secondary physiologic factors and not to primary oxidation.

Summary. Evidence obtained from an experiment with rats of the Long-Evans strain suggests that mid-lethal whole body X-irradiation (710 r) may directly reduce tissue ascorbic acid. In rats of the Wistar strain, however, except for the adrenal glands, a higher dose (1000 r) did not result in an immediate reduction in tissue ascorbic acid but rather a tendency to increase was noted. In both strains, the effect of X-irradiation on the adrenal ascorbic acid could be ascribed to activation of the pituitary. In the Wistar strain animals, the changes in the ascorbic acid of the liver and blood could also be explained by this mechanism.

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Received July 23, 1953. P.S.E.B.M., 1953, v84.

Action of Nicotinic Stimulant Agents on Rabbit Skeletal Muscle I. Nicotine and Acetylcholine. (20682)

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Langley and Dickinson(1) preventing nicotine tremors by section of the motor nerves in *anesthetized mammals* gave evidence of their central origin; this locus of action was confirmed by Amantea(2) and Galamini(3), who noted motor stimulation after cortical application of nicotine, and by Longo and Bovet(4), who recorded brain waves similar to *grand mal* seizures in curarized rabbits receiving repeatedly high doses of nicotine. Nevertheless, the peripheral stimulant effect of nicotine(5,6) and acetylcholine(7) on mammalian skeletal muscle is also unequivocally established.

Our recent observations of the blockade by various neuromuscular agents(8), and ether (9) of nicotine-induced tremors in unanesthetized rabbits prompted a reinvestigation of the problem in *unanesthetized unmedicated animals*.

Materials and methods. The rabbit was selected as the most suitable animal for study in the unanesthetized state. Eighteen male albino New Zealand rabbits, weighing between 1.5 and 2.5 kg were used in 30 assays. One hindleg was denervated by section of the sciatic nerve, while the contralateral leg in the same animal was used as control. All animals were tested 30 minutes after denervation. The survivors, however, were tested again 3, 5, 8, and 10 days after denervation. Both early and late tests were considered. For testing muscle tremors, the same procedure as described by Longo and Bovet(10) was used, with the addition that independent movements of both hind legs were recorded simultaneously. For recording individual isotonic muscle contractions, rabbits were prepared as follows. The Achilles tendon was exposed, severed just proximal to the calcaneum, and securely tied by a nylon thread to an isotonic muscle lever. The leg was immobilized by clamps holding a rod drilled through the lower part of the femur.* For experiments with ether the sectioned tibial nerve, exposed just below the knee, was stimulated by means of shielded electrodes. Stimulation was provided by an electronic square wave "Electrodyne" at a voltage of 200 and a frequency of 0.5 pulse per second. Nicotine tartrate, 0.5% aqueous solution, was injected at a dose of one mg/kg into the marginal ear vein. Acetylcholine (10 μg/kg) was injected by the same route 10 minutes after intravenous injection of 10 mg/kg atropine sulfate and 5 minutes after one mg/kg physostigmine salicylate. Ethyl ether (for anesthesia) was given by inhalation in anesthetic concentrations.

Results. Effects of nicotine upon denervated rabbit muscle. No significant difference was noted in the results whether experiments were conducted either immediately after or some days after the section of the nerve. When tremors induced by a small dose of nicotine in the denervated hindleg were compared with those recorded in the contralateral normal leg of the unanesthetized rabbit, a positive response was noted in both legs in 6 out of 6 experiments; of these the amplitude and duration were either the same (3 cases) or greater in the denervated hindleg (Fig. 1). In 19 experiments, isotonic muscle contractions induced by nicotine were compared in both hindlegs. In 14 animals the response was the same in both hind legs. In 4 the response was smaller in the denervated leg (Fig. 2), and in one it was even greater. A longer induction period was noted in the denervated leg than in the normal one.

Effect of acetylcholine in the atropinized-eserinized rabbit. In 5 experiments, acetylcholine induced contractions of about the same magnitude in the denervated and the normal contralateral leg muscle.

Effects of ether on nerve muscle preparation of the rabbit. Light anesthesia induced

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* We are indebted to Dr. Walter Riker for demonstration of this method.

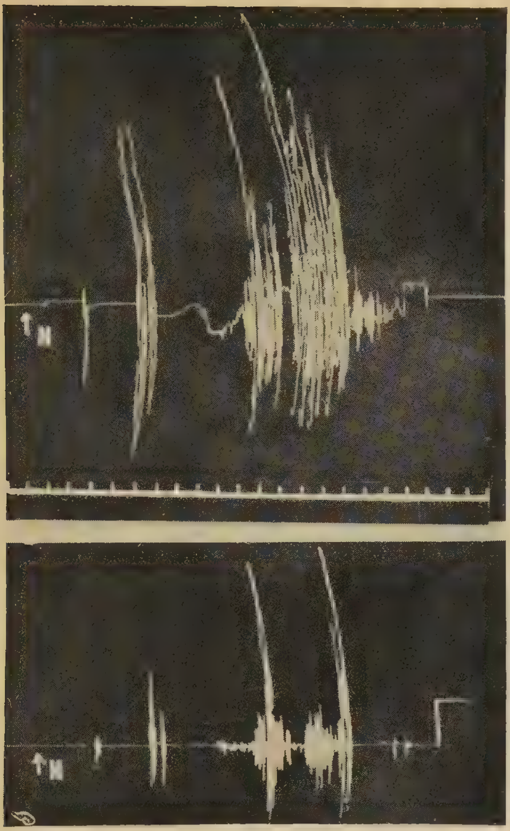

FIG. 1. Comparative tremors induced by nicotine in acutely denervated hind leg (upper tracing) and contralateral normal leg (lower tracing) of an unanesthetized rabbit. Time in 5 sec. N: Nicotine tartrate 1 mg/kg inj. into ear marginal vein.

by ether inhalation caused a temporary excitation followed by a decrease of approximately 80% in the response to sciatic stimulation. This effect was reversible and disappeared gradually after removal of the anesthetic.

Discussion. Our data, which give evidence that in the unanesthetized rabbit nicotine induced tremors and muscle contractions are not prevented by acute or chronic section of the motor nerves, do not agree with the results obtained in *anesthetized mammals* by Langley and Dickinson(1) who state, "After section of the nerves which run to any part of the body, we have not seen either a movement or twitching take place in this part on injecting nicotin. . . . It will be remembered that in the frog nicotin has a slight peripheral action and thereby causes a faint fibrillar twitching of the muscles." Undoubtedly, their results were conditioned by the use of ether which was omitted in our experiments. It seems reasonable to suggest that ether may interfere with the effect of nicotine at the motor endplate of the neuromuscular junction. This suggestion is in accord with earlier data which gives evidence of the neuromuscular blocking effect of ether, either alone in mammals (11,12), and in man(13), or in enhancing d-tubocurarine in animals(14-16), and in man(17). Such a mechanism for the action of ether would account for our earlier observation of the ether blockade of nicotine induced tremors(9). Besides the central stimulant effect of nicotine, which was recently reemphasized by Longo and Bovet(4), one must conclude that nicotine exerts a separate peripheral action. This fact should be considered in the interpretation of the blockade of nicotine-induced tremors in screening anti-Parkinsonism drugs.

Summary. In the *unanesthetized rabbit,* tremors of the hind legs and muscle contractions of the gastrocnemius muscle induced by intravenous doses of nicotine tartrate or acetylcholine are not prevented by acute or chronic section of the sciatic nerve. It is concluded that nicotine exerts, in addition to a central action, a separate and distinct

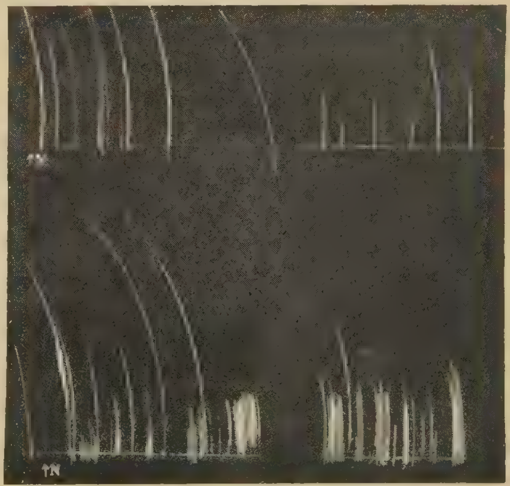

FIG. 2. Comparative isotonic muscle contractions induced by nicotine in acutely denervated gastrocnemius (upper tracing) and contralateral normal muscle (lower tracing). N: Nicotine tartrate 1 mg/kg inj. into ear marginal vein.

peripheral effect on mammalian striated muscle.

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Received July 30, 1953. P.S.E.B.M., 1953, v84.

Relative Inability of Heparin to Prolong Clotting Time of Oxalated and Resin-Treated Blood and Plasma.*† (20683)

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In the course of studies on the anticoagulant activity of heparin it was noticed that this drug failed to prolong the clotting time of recalcified, oxalated or resin-treated blood and plasma. In view of the possible importance of this phenomenon as a source of error in the evaluation of the anticoagulant activity of heparin, it was decided to investigate the matter further.

Materials and methods. A) *Collection of blood and plasma in various anticoagulants.* Blood was collected from healthy donors in Silicone-coated glass syringes through Arquad 2-C‡ coated needles. It was transferred to Silicone-coated test tubes and made incoagulable by the addition of one of the following reagents: (a) sodium or potassium oxalate, 0.1 M; (b) sodium citrate, 0.2 M; (c) Sequestrene-Na_2§ 1% in 0.7% NaCl, all in the volume of one ml to 9 ml of blood. In other experiments, whole blood was decalcified by passage through a column of cation-exchange resin (2.5 g of resin to 10 ml of blood) with the technic described by one of us(1). Dowex-50‖ and IR-100¶ in the sodium cycle were used in this work. In all cases plasma was separated by centrifugation of the whole blood at 2000 rpm for 10 minutes. B) *Addition of heparin.* Saline solution containing various amounts of heparin was added, in the

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* Supported by grant-in-aid from the American Heart Assn.

† The authors wish to express their appreciation to Dr. Peter Bernfeld and to Mr. Mordecai Berkowitz, Cancer Research Unit, Tufts College Medical School, for repeating and confirming some of the results reported.

‡ Dicoco-dimethyl-ammonium chloride; available from Armour Co., Chicago, Ill.

§ Disodium ethylendiamine-tetracetate-dihydrate; available from Alrose Chemical Co., Providence, R. I.

‖ Available from Dow Chemical Co., Midland, Mich.

¶ Available from Rohm and Haas Co., Philadelphia, Pa.,

TABLE I. Clotting Time of Recalcified Plasma, in Presence of Various Amounts of Heparin Sodium (Average Values of Several Experiments). [Plasma was obtained by centrifugation from blood made incoagulable by various technics.]

Anticoagulant originally used (final conc.)	Sodium oxalate, .01 M	Sodium citrate, .02 M	Sequestrene-Na_2, 0.1%	Dowex-50	IR-100	Native plasma*
Heparin, γ/ml	Clotting times (min.)					
0	3.5	4	4.5	3	3.5	4
1	3.5	14	12.5	3.5	4.5	15.5
2	4	26	29	3.5	4	26
3	4	62	58	4	5.5	56
4	4.5	∞	∞	7	6	∞
5	5.5	∞	∞	8.5	8.5	∞
10	7	∞	∞	12	11.5	—
20	12.5	—	—	17	19	—
30	∞	—	—	∞	∞	

* No recalcifying agent added.

volume of 0.1 ml for every 0.9 ml of blood or plasma. C) *Determination of clotting time of recalcified blood and plasma.* Blood or plasma made incoagulable by the technics described under A), with or without the addition of heparin, were transferred to glass test tubes in water bath at 37°C and recalcified with 1/10 volume 0.2 M $CaCl_2$. The test tubes were then tilted every 30 seconds for 10 minutes and every minute thereafter, until they could be inverted without escape of content. D) *Determination of prothrombin time* was carried out by the one-stage method of Quick, using dehydrated human brain thromboplastin.

Results. Heparin and clotting time of recalcified blood and plasma collected in various anticoagulants. Table I shows that as much as 5 γ of heparin sodium per ml failed to prolong very significantly the clotting time after recalcification of plasma from blood made incoagulable by addition of sodium or potassium oxalate or by passage through cation-exchange resins. This result was in sharp contrast with that obtained with native, citrate or Sequestrene-Na_2 treated plasma. In the latter case, the clotting time after recalcification was normally retarded by the addition of heparin. The effect of oxalate and resins was not modified by changing the concentration of $CaCl_2$ used to recalcify the mixture, although all clotting times were uniformly delayed when the concentration of $CaCl_2$ was over 0.02 M in the final mixture. Essentially similar results were obtained when whole blood instead of plasma, dog and rabbit blood or plasma were used in similar experiments.

Clotting time of recalcified plasma collected by various technics from patients receiving heparin. The phenomenon described was studied in plasma and blood collected from patients receiving intravenous heparin. Doses of one mg/kilo weight were being administered for therapeutic purposes. Blood was collected 10 minutes after the injection of the full dose of the drug (to allow maximum anticoagulant effect) and made incoagulable by any of the technics described in A). It was again noticed that, on recalcification, samples containing sodium oxalate or passed through cation-exchange resins would clot

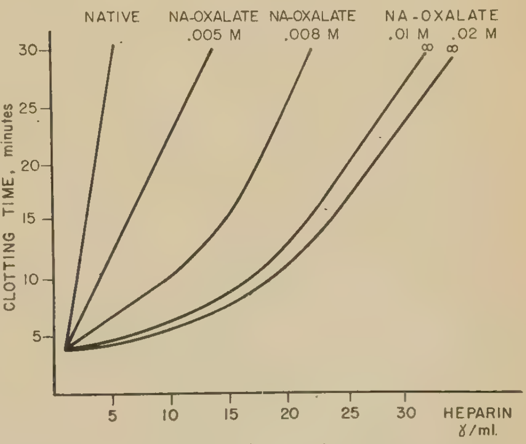

FIG. 1. Relationship of heparin concentration to clotting time of native or recalcified plasma (made incoagulable by addition of various concentrations of calcium oxalate). Plasma was recalcified with concentrations of $CaCl_2$ equimolecular to that of the sodium oxalate used as anticoagulant.

TABLE II. Prothrombin Times (One Stage) of Samples of Oxalated and Citrated Plasma in Presence of Heparin (Avg 2 Exp.).

Heparin sodium	γ/ml	0	1	5	10
Anticoagulant in plasma		Prothrombin time (sec.)			
Sodium oxalate	.01 M	13.0	14.5	19.0	25.4
" citrate	.02 M	12.5	13.5	15.0	19.5

much faster than samples collected in sodium citrate or Sequestrene-Na$_2$.

Quantitative aspects of the sodium oxalate-heparin antagonism. As indicated in Fig. 1, the anticoagulant activity of heparin became more pronounced as the concentration of sodium oxalate was decreased below the optimum level.

Prothrombin time of plasma from blood made incoagulable with various technics, in the presence of heparin. Blood was collected from healthy donors as described and made incoagulable by the addition of sodium citrate 0.02 M or sodium oxalate 0.01 M. Various amounts of heparin were added and the prothrombin time determined by the one-stage method. A similar trend was noticed for both citrated and oxalated plasmas (a behavior completely opposed to the conduct observed for whole blood and plasma clotting time), although the prothrombin time of the citrated sample was uniformly shorter (Table II).

Discussion. The most significant finding of this work is that the anticoagulant activity of heparin was limited when the drug was added to blood or plasma which had been made incoagulable by decalcification with addition of sodium oxalate or passage through cation-exchange resins. The phenomenon was consistently observed and clearly reproducible when sodium oxalate was the anticoagulant used. When cation-exchange resins were used, however, we often observed that excellency of technic in venipuncture and the speed with which the blood was passed through the resin would change somewhat the values obtained; the anticoagulant effect of heparin becoming more pronounced if blood was collected with trauma to the tissue and was not promptly passed through the resin. The phenomenon was not related to changes in the pH of the plasma due to the various technics of decalcification, since direct measurements of the pH showed that this remained relatively constant.

There is obviously a relationship between mechanism of decalcification and anticoagulant activity of heparin. The mechanism through which sodium oxalate causes incoagulability of blood is apparently different from that of sodium citrate (and according to our experience, Sequestrene-Na$_2$) (2,3). The heparin-resistance of oxalated blood (directly proportional to the concentration of oxalate used) when compared to the heparin-sensitivity of citrated and Sequestrene-Na$_2$ treated blood could be related to the different mechanism of decalcification of these anticoagulants. One wonders whether the presence of calcium, combined or free, is necessary to the anticoagulant activity of heparin, or whether oxalate can directly inhibit the activity of the anticoagulant.

Whatever the explanation of these findings, it should be emphasized that not cation-exchange resin or sodium oxalate treated but only native, sodium citrate or Sequestrene-Na$_2$ treated blood and plasma are suitable for studies of the anticoagulant activity of heparin *in vitro*.

Summary. Plasma decalcified with sodium oxalate or passage through cation-exchange resins is relatively resistant to the anticoagulant effect of heparin on recalcification. Native plasma, and sodium citrate or Sequestrene-Na$_2$ tested plasma react normally to heparin. The latter plasmas only should be used when testing the anticoagulant effect of heparin *in vitro*.

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Received August 3, 1953. P.S.E.B.M., 1953, v84.

Further Studies on Renal Toxicity of PGA.* (20684)

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The occurrence of renal lesions in guinea pigs given folic acid (pteroylglutamic acid, PGA) parenterally has been reported previously from this laboratory(1). The present investigation was undertaken to study the possibility that photolytic products of PGA(2,3) might have been present in the solutions of PGA used and have produced these renal lesions. In addition, it was planned to test the impression that vit. C enhanced the resistance of the guinea pig to this form of renal injury.

Methods. The scorbutigenic diet and the experimental routine have been described(1). The two preparations of PGA used for injections were prepared from crystalline PGA‡ as follows: 1) Crystalline PGA was recrystallized twice from hot water, dissolved in 0.2 M phosphate buffer of pH 7.5 in a concentration of 8 mg/ml and kept in the dark. This PGA was recrystallized a third time every other day during the course of the experiment. Analyses for diazotizable amine(4) revealed a concentration of less than 0.013 mg/ml (equivalent to 0.16% PABA) 2 days after the final recrystallization. This solution will be referred to as recrystallized PGA. 2) A solution of PGA containing 8 mg/ml in the same buffer was exposed to sunlight in a clear glass bottle for several days before injections were begun and then kept in the dark. Diazotizable amine was present in this solution at a concentration of 12 mg % (1.5% as PABA). This preparation will be called irradiated PGA. Following a 10-day conditioning period on the scorbutigenic diet supplemented with 5 mg of ascorbic acid daily, the guinea pigs were divided into experimental groups receiving daily subcutaneous injections into the abdominal wall of 1 ml of a PGA preparation (8 mg PGA) or control injections of 1 ml of the phosphate buffer. Half the animals in each group were given 2 mg of ascorbic acid daily and this vitamin was withheld from the remainder. These guinea pigs weighed 350-450 g, and all but 3 were females. After 10 daily injections the surviving animals were killed by a blow on the head, their kidneys removed, weighed, and sections fixed in Bouin's fixative were stained with hematoxylin and eosin.

Results. Gross pathological examination revealed changes suggestive of scurvy in only one of the animals fed the scorbutigenic diet without supplementary vit. C. Scarring and hematomata were present in the abdominal walls of the animals receiving PGA in either form. An apparent increase in size of the kidneys and mottling of the surface were frequently noted in both groups receiving PGA. The number of animals surviving for 10 days, their change in weight, the weight of their kidneys, and the incidence of the microscopic changes(1) previously reported are given in Table I.

All of the animals receiving either recrystallized or irradiated PGA had microscopic renal lesions.§ The lesions produced by the irradiated PGA could not be differentiated from those produced by recrystallized PGA. No differences in the severity of the microscopic changes, weight changes or kidney weights could be attributed to the presence or absence of ascorbic acid in the diet. The animals receiving either solution of PGA lost more weight than the control group ($P < 0.05$) and their kidneys were heavier on both an absolute ($P < .001$) and relative basis ($P < .01$). The

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* This work was supported by grants from the National Vitamin Foundation, and the U. S. Public Health Service.

† National Research Council Fellow. Present address: Naval Medical Research Institute, Bethesda, Md.

‡ Supplied by Dr. T. H. Jukes of Lederle Laboratories, Inc.

§ The assistance of Dr. James R. Dawson, Department of Pathology, University of Minnesota Medical School, and Dr. John Shapiro, Department of Pathology, Vanderbilt University School of Medicine, in the interpretation of these sections is gratefully acknowledged.

TABLE I. Comparison of Renal Effects in the Guinea Pig of Recrystallized and Irradiated PGA.

	Ascorbic acid, mg/day	No. of 10-day survivors/No. in group	% incidence microscopic renal lesions	Change in body wt in 10 days	Kidney wt of 10-day survivors, %/body wt	Kidney wt of all animals at death
				(g)		(g)
Controls	2	5/5	0	+ 1 ± 18	.89 ± .04	2.62 ± .15
	0	5/5	0	— 3 ± 8	1.01 ± .03	2.88 ± .09
Irradiated PGA,	2	8/10	100	—32 ± 18	1.38 ± .16	3.34 ± .10
8 mg/day	0	7/10	100	—29 ± 19	1.15 ± .10	3.40 ± .16
Recrystallized PGA,	2	6/10	100	—22 ± 12	1.29 ± .13	3.62 ± .19
8 mg/day	0	6/10	100	—40 ± 14	1.41 ± .14	3.52 ± .18

TABLE II. Effectiveness of Minimal Dosages of PGA in Production of Renal Lesions in the Guinea Pig.

PGA, mg/day	Renal lesions per No. of animals	6-day wt change, g
.5	2/5	+15 ± 5
1.0	1/5	+16 ± 7
2.0	1/5	— 2 ± 8

greater mortality noted in the group receiving the recrystallized PGA was not significant.

An attempt was made to determine the minimal subcutaneous dose PGA which would produce these lesions. Groups of 5 guinea pigs weighing 250-300 g received the scorbutigenic diet without added ascorbic acid and were each given 0.5, 1, or 2 mg of recrystallized PGA subcutaneously daily for 6 days and killed on the seventh day. Results are shown in Table II. Although 4 animals showed the renal lesions, they were not as pronounced as those noted in the previous experiment. The differences between the weight changes of the 3 groups were not significant. These findings indicate that the renal lesions appear regularly only when amounts of PGA greater than 2 mg per day for 6 days are administered, and that the lesions are observed regularly when the dose is 8 mg daily for 10 days. The minimal effective dose in reversing the hydroxyphenyluria in the scorbutic tyrosine-fed guinea pig lies between 2.0 and 5.0 mg of PGA per day(5). These doses of PGA, approximately 8-20 mg per kg of body weight, correspond to the amounts effective in reversing the tyrosyluria of scorbutic infants(6) or in producing hypertension in rats(7). Similar renal lesions were observed in association with the latter effect(7), although no changes in kidney weight were found. Blood pressure measurements have not been made on guinea pigs treated with PGA.

Summary. 1. The photolytic oxidation products of PGA could not be implicated in the production in guinea pigs of renal lesions by large parenteral doses of PGA. 2. These lesions were uniformly produced in guinea pigs by the daily administration of 8 mg of PGA (20 mg of PGA/kg) for periods of 6-10 days, and they occurred in occasional animals at a daily dosage of 2 mg (8 mg/kg). 3. Ascorbic acid did not appear to be a factor in the production of the lesions.

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Received August 6, 1953. P.S.E.B.M., 1953, v84.

Effect of Phenylbutazone (3,5-dioxo-1, 2-diphenyl-4-n-butyl-pyrazolidin) on Renal Function. (20685)

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Phenylbutazone, a potent analgesic, anti-inflammatory agent, has had extensive use in patients with painful disorders of joints. Up to 20% of patients receiving this drug have been noted to develop edema, the actual number apparently depending upon the vigor of scrutiny. It has been demonstrated that retention of salt and water with accompanying weight gain is an almost constant, immediate, and sustained effect which is not mediated through reduction in glomerular filtration rate (1,2). Only 2 of 18 patients carefully followed at this hospital failed to gain weight. That the effect is immediate can be demonstrated by a sharp reduction in urine output and an average weight gain of 2 lb during the first 24 hours of therapy. This continues daily for 5 or 6 days until the patient has gained an average of 10 to 16 lb. It is sustained until the drug is stopped, at which time a spontaneous diuresis with weight loss occurs. The present study was undertaken in an attempt to clarify further the mechanism of this salt and water retention.

Materials and methods. The renal clearance of inulin (C_{IN}) and para-aminohippurate (C_{PAH}) was determined in 8 patients before and after institution of Phenylbutazone therapy. In 4 of these, maximum tubular excretion of para-aminohippurate (Tm_{PAH}) was also done. The patients were males 18 to 46 years of age who were admitted to the hospital for treatment of arthritis. Four of these patients had rheumatoid spondylitis; 3 had early minimal to moderate rheumatoid arthritis; and 1 patient had gout. Phenylbutazone therapy consisted of 800 mg daily, orally, in 4 equal doses in all except 1 patient who received 1 g daily, intramuscularly. The clearances were done in the morning postabsorptive state following liberal water ingestion to insure adequate urine flow. The clearance technics employed were those used by Goldring and Chasis (3). Clearances were performed on the 3rd or 4th day of Phenylbutazone therapy since the peak of salt and water retention effect seemed to occur at this time. Six additional patients free of renal disease and receiving para-aminosalicylic acid in doses of 12 g daily, orally, for pulmonary tuberculosis were selected for study. Oral therapy was replaced by 10 g of PASA intravenously, given over a period of 30 minutes at a recorded time each morning for 5 mornings. The intravenous route was chosen to assure complete absorption and to avoid the complications of nausea and vomiting which sometimes occur during oral therapy. At exactly 4 and 7 hours after the initiation of intravenous infusion, samples for the determination of PASA blood levels were drawn. Phenylbutazone, 1 g daily, intramuscularly, was given on the evenings pre-

TABLE I. Renal Clearances before and during Phenylbutazone Therapy.

	Inulin clearance, cc/min.*		PAH clearance, cc/min.*		TmPAH, mg/min.*	
	Before	During	Before	During	Before	During
T.	113†	117	595	347		
	100†	105	—	339		
S.	147	145	782	501		
	179	115	1354	427		
T.	123	117	945	321		
	104	91	782	299		
G.	132	88	591	533		
	128	86	601	489		
	100	—	510	—		
L.	143	102	907	496	—	
	131	93	838	567	—	
	150	103	—	—	133	32
D.	132	145	916	465	—	
	124	122	961	449	—	
	127	132	—	—	110	24
F.	130	143	959	724	—	—
	153	125	1108	605	—	—
	126	140	—	—	137	68
S.	151	144	996	558	—	—
	141	147	878	627	—	—
	133	133	—	—	131	26

* Not corrected for body surface area.

† Consecutive vertical figures for each patient represent consecutive 15 min. urine collection periods.

ceding the 3rd, 4th, and 5th day of this regimen. PASA blood levels were determined by the Method of Marshall(4).

Results. Table I summarizes the results of the renal clearances of inulin (C_{IN}) paraaminohippurate (C_{PAH}), and the maximum tubular excretion of PAH (Tm_{PAH}) in the 8 patients studied. There is no significant change in the glomerular filtration rate. However, it will be noted that there is a constant reduction of C_{PAH} in all except 1 patient (G). This patient is the only one of the group who had gouty arthritis. In the 4 patients in whom Tm_{PAH} was determined before and during Phenylbutazone administration, a marked and constant decrease of 50% or more is noted. All of these patients gained from 10 to 16 lb of weight during the first week of Phenylbutazone therapy. Only 2 developed actual edema. In S., this was manifested as morning puffiness of the face and periorbital swelling. F. T. developed swelling of the ankles toward the end of the day.

Chart I plots the fall in PASA blood levels from the fourth to seventh hours following intravenous injection of this drug. Table II tabulates a 2-day average for PASA blood levels before institution of Phenylbutazone and a three-day average during Phenylbutazone administration in 6 patients. No real difference in the levels before and during Phenylbutazone is apparent. Due to the intravenous route of administration, levels are high and the fall rapid. The small differences noted are within the range of error of the method used.

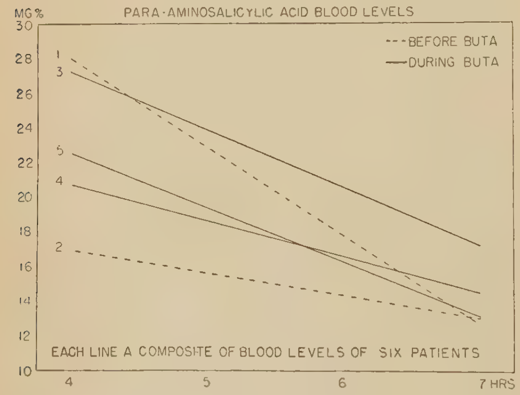

CHART I. Effect of phenylbutazone on "para-aminosalicylic acid blood levels." Numbers 1 through 5 refer to consecutive days of study. Phenylbutazone started on 3rd day.

TABLE II. Para-aminosalicylic Acid Blood Levels before and during Phenylbutazone Rx.

Patient	Hour*	Before,† mg %	During,‡ mg %
R.	4th	24.1	25.1
	7th	15.4	16.0
G.	4th	25.8	24.8
	7th	15.5	18.2
C.	4th	16.5	15.5
	7th	12.1	12.1
S.	4th	23.5	23.6
	7th	14.7	15.9
G.	4th	18.1	19.4
	7th	12.6	13.1
M.	4th	23.8	25.8
	7th	12.2	15.8

* Hours following PASA intravenously administered.

† Each figure a composite of 2 days blood levels.

‡ " " " " " 3 " " " .

Discussion. The reduction of C_{PAH} by Phenylbutazone in the first 4 patients suggested that a reduction had been effected in renal plasma flow. However, a specific inhibition of tubular excretory activity was quite possible and could not be excluded, except by extraction studies or quantitative excretory studies.

It is believed that the marked reduction noted in Tm_{PAH} during Phenylbutazone administration confirms an inhibition of tubular excretory activity. The knowledge that another tubular depressant (Benemid) decreased the secretions of both para-aminohippurate and para-aminosalicylic acid(5) led us to study the effect of Phenylbutazone on PASA excretion also. It is surprising that there was no apparent inhibition of PASA excretion. It must be admitted that a 2-point curve of 4 and 7 hours is not complete, yet Benemid effects are conclusive when so plotted. One might speculate that the effect of Phenylbutazone on PAH excretion is not that of inhibition of a selective tubular transport mechanism.

There is mounting evidence that the effect of Phenylbutazone is a more general one of depression of cellular metabolism. Kuzell reported that this drug inhibited oxygen consumption of rat cerebral tissue slices in direct proportion to its concentration(6). Werner reports a personal communication from Engleman that this drug inhibits transiently the accumulation of I^{131}(7). Certainly, cellular respiratory studies are in order, and renal oxygen uptake determinations before and during Phenylbutazone administration would be helpful.

Excretory studies of other renal tubular substances would be enlightening, especially of sodium. From clinical studies, it might be assumed that tubular reabsorption of sodium is increased. If so, this is indeed paradoxical since mercurial diuretics inhibit tubular excretion of PAH and decrease reabsorption of sodium.

Conclusions. 1) Phenylbutazone causes retention of salt and water in approximately 80% of patients receiving this drug with clinical edema becoming manifest in approximately 10%. 2) Phenylbutazone markedly depresses renal tubular excretion of para-aminohippurate. 3) Excretion of para-aminosalicylic acid does not appear to be inhibited by this drug. 4) Further studies are needed to clarify the relation, if any, between decreased excretion of para-aminohippurate and the clinically observed increased reabsorption of sodium.

The author wishes to express his appreciation for the technical assistance of Dr. Legh R. Scott and Miss Ann Payne.

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Received September 14, 1953. P.S.E.B.M., 1953, v84.

Effect of Ethionine and Other Materials on Semliki Forest Virus Infection in the Mouse.* (20686)

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Semliki forest virus was isolated by Smithburn and Haddow(1) from mosquitoes collected in Uganda. Although the virus has not been recovered from man, antibodies for it have been demonstrated in human serum specimens collected in Uganda and in India (2,3). In mice, the virus produces a fatal disease following inoculation by the intracerebral, subcutaneous, intramuscular or intraperitoneal routes(1). Animals inoculated intracerebrally with the virus usually die within one week and within 2 weeks after intraperitoneal inoculation. Paralysis of one or both posterior extremities usually constitutes the first sign of infection but hyperirritability and convulsions may occur. Animals may recover from paralysis.

Since the peripheral inoculation of mice with Semliki forest virus produces a disease involving the central nervous system, this virus provides a useful means for the investigation of materials which may alter resistance of the mouse to neurotropic viruses. The Semliki forest-mouse model has been used in a study involving a number of amino acids, purines, pyrimidines, and thiosemicarbazones. The most significant results were obtained with DL-ethionine and 5-phenoxythiouracils.

Materials and methods. The strain of Semliki forest virus used in these experiments was obtained in 1951 from Dr. H. M. Powell of Lilly Research Laboratories who, in turn, had received it from Dr. Smithburn. Five intracerebral passages in mice have been

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* This work was supported by a contract between the Office of Naval Research, Department of the Navy, and Indiana University and by a grant from Burroughs-Wellcome & Co.

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TABLE I. Effect of Ethionine and Other Materials on the Course of Semliki Forest Virus Infection in the Mouse.

Compound	Treatment			Proportion of inoculated mice surviving infection	
	Dose*	Route	Days	Treated	Untreated
Virus inoculated intracer.					
DL-e†	.15	Diet	–3 to 6	33/64	33/68
DL-e	20–30	I.P.	–1, 0 only	3/20	2/24
Virus inoculated intraper.					
D-e	.15	Diet	–3 to 6	20/27	8/24
L-e	.15	"	"	19/30	8/24
DL-e	.15	"	"	205/378	111/413
DL-e	.15	"	"	13/20	8/20
DL-e	.15	"	–3 to 1	11/20	8/20
DL-e	.15	"	0 to 2	7/19	8/20
DL-e	.3	"	"	11/19	8/20
DL-e	20–30	I.P.	–2 only	3/10	3/10
DL-e	20–30	"	–1 "	26/39	13/40
DL-e	20–30	"	0 "	22/39	14/40
DL-e	20–30	"	1 "	7/29	9/29
DL-e	20–30	"	2 "	5/20	7/20
DL-m‡	1.0	Diet	–3 to 6	20/59	16/59
DL-e	.15	"	"	27/56	16/59
DL-m and DL-e	1.0 and .15	"	"	22/60	16/59
Dichlorophenoxy thiouracil	.75	"	"	16/40	8/55
Adenine sulfate	.03	"	"	8/15	8/17
Benzimidazole	.1	"	"	3/16	7/16
Purine derivatives§	.02–.1	"	"	23/115	57/130
Thiosemicarbazones‖	.03–.08; 5	I.P.	"	18/69	17/66
Atabrine hydrochloride	.05	Diet	–2 to 6	7/22	14/26
Antibiotic M5-8450		I.P.	–3 to 6	9/10	4/10

* Materials administered in diet are listed in terms of concentration (%) and those given intraperitoneally (I.P.) in mg/dose.

† Ethionine.

‡ Methionine.

§ Purine derivatives: 2,6-diamino, 6-mercapto, 2-mercapto-6-amino, 2,6,8-dithiohydroxy, 2-amino-6,8-dimercapto, 6-hydroxy-8-mercapto, 1,3-dimethyl-2,6-dimercapto, 6-decylamino, 6-methyl, and 2,6-diamino-8-azapurine; 2-thioxanthine, 8-azaxanthine and thioguanine.

‖ Thiosemicarbazones: isatin, cinchoninaldehyde, 5-bromo-2-thenaldehyde, benzaldehyde and *o*-methoxybenzaldehyde.

made in this laboratory. Pooled infected brain tissues from the last 2 transfers served as the inoculum for experiments to be reported. In most experiments, mice were injected intraperitoneally with 0.1 ml of a 10^{-5} dilution of infected brain tissue prepared in Locke's solution containing 5% normal rabbit serum. This inoculum contained approximately 600 intracerebral LD_{50} doses of virus. In a few experiments, mice were inoculated intracerebrally with 0.03 ml of a 10^{-6} dilution of infected tissue. This inoculuum contained approximately 18 intracerebral LD_{50} doses of virus. Inoculated animals were observed for 16-20 days. Materials to be administered in the diet were ground in a mortar and mixed in the basal casein-sucrose diet(4) to give the desired concentrations. Substances to be injected intraperitoneally were dissolved or suspended in sterile distilled water, the pH adjusted to approximately 7.0, and the desired quantity of compound injected in a volume of 0.5-0.8 ml.

Results. When Semliki forest virus was inoculated intracerebrally into mice, DL-ethionine failed to protect animals (Table I). When the virus was injected intraperitoneally, however, treatment with ethionine was observed to reduce the fatality rate. In 31 experiments in which DL-ethionine was fed in the diet in a concentration of 0.15%, 205 of 378 treated mice (54%) survived as compared to 111 of 413 (27%) in the corresponding untreated groups. Both the D- and L-forms of ethionine protected mice against the virus. When DL-ethionine was administered intraperitoneally in a single dose of 20-30 mg, either on the day preceding or within 1-2 hours after viral inoculation, the survival rate of mice was increased. Protection was not obtained when the compound was injected either 2 days before or one or 2 days after initiation of infection.

Ethionine treated mice which survived infection by Semliki forest virus usually were found to have developed immunity against the virus. All of 15 survivors treated with a single intraperitoneal injection of ethionine within 2 hours after viral inoculation were found to be completely resistant to the same dose of virus given by the same route 2 weeks later. Thirteen of 16 survivors treated with a single intraperitoneal injection of ethionine 24 hours before viral inoculation survived a challenge dose of approximately 18 intracerebral LD_{50} doses of virus given intracerbrally 2 weeks later.

Since ethionine is a structural analogue of methionine, experiments were carried out to determine whether methionine would counteract the protective action of ethionine when both materials were fed in the diet. The combined data from 6 experiments are presented in Table I. In some experiments, the number of surviving mice fed a combination of methionine and ethionine was similar to that of untreated groups while in other experiments methionine appeared to have no effect. A concentration of 2% of methionine in the diet did not produce a result different from that obtained with 1%. Folic acid (10 mg %) and vit. B_{12} (0.005 mg %) in combination with ethionine or with ethionine and methionine or ethionine and homocysteine gave essentially the same results. DL-cystine (2%) was not observed to overcome the protective action of ethionine.

Dichlorophenoxy thiouracil (5-(2′4′-dichlorophenoxy-4-hydroxy-2-mercaptopyrimidine)) when fed in the diet in a concentration of 0.5-0.75% increased the number of survivors (Table I). Similar results were obtained with 4′-bromo, 3′4′-dicholor and 3′4′-dimethyl derivatives of 5-phenoxy thiouracil. In 8 experiments with this group of compounds, 26 of 64 treated mice survived in contrast to 12 of 87 untreated controls.

Antibiotic M5-8450‡ protected mice against Semliki forest virus. Treatment consisted of the intraperitoneal injection of an arbitrary amount of the antibiotic for 3 days before and 6 days after viral inoculation.

The adenine antagonists, benzimidazole and 2,6-diaminopurine, tended to increase susceptibility of mice to Semliki forest virus. Other purine derivatives exhibited a similar effect. In 14 experiments with 13 purine derivatives, 23 of 115 treated animals (20%) survived in contrast to 57 of 130 mice in untreated groups (43%). None of 5 thiosemicarbazones tested altered susceptibility of mice to the virus.

Tests with other materials. Little or no influence on the course of Semliki forest viral infection in mice was observed as the result of feeding the following materials in the concentrations listed: Acetyl (0.5%), alanyl (0.15%), benzoyl (0.2%), propionyl (0.5%), sulfone (0.5%), and sulfoxide (0.5%) derivatives of methionine; *alpha* amino butyric acid (1%), *alpha* hydroxy *gamma* methyl mercapto butyric acid (0.2%), 5-methyl tryptophane (0.09%), N-2-carboxy-ethyl-tyrosine (0.15%), *p*-chloro (0.5%), and *p*-bromophenylalanine (0.5%), *beta* totylalanine (0.3%), *beta*-3-thienylalanine (0.1%), phenylserine (0.5%), aureomycin (0.1%), and chloromycetin (0.25%).

Discussion. Ethionine has been reported to inhibit multiplication of mouse encephalomyelitis, influenza (type A) and poliomyelitis (type 2) viruses *in vitro*(5-7). Methionine counteracted the inhibition of growth of influenza and poliomyelitis viruses produced by ethionine(6-7). Data have been presented which demonstrate that ethionine increases the survival rate of mice inoculated intraperitoneally but not of animals inoculated intracerebrally with Semliki forest virus. The compound is effective either when fed in the diet or injected by the peritoneal route. Treatment of mice with ethionine is most effective when the compound is given within

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‡ Obtained through the courtesy of Dr. H. M. Powell, Lilly Research Laboratories, Indianapolis, Ind.

24 hours preceding the inoculation of virus. Ethionine treated mice which survive Semliki forest viral infection usually are immune to the virus. Although supplementing an ethionine-containing diet with methionine appeared to reduce the protective capacity of ethionine in some experiments, the results obtained were variable and are not interpreted as a true reversal.

Dichlorophenoxy thiouracil likewise was observed to increase the survival rate of mice inoculated with Semliki forest virus. Isatin thiosemicarbazone, however, was ineffective. Both of these compounds protect mice against vaccinia virus(4,8). Antibiotic M5-8450 and the closely related or identical compound, Helenine, protect mice not only against Semliki forest virus(9,10), but also against MM and Columbia SK strains of encephalomyelitis virus(9,11), and the MEF1 strain of poliomyelitis virus(12). 2,6-Diaminopurine, which reduces the fatality rate of mice inoculated with Russian Far East encephalitis virus(13), increases the susceptibility of animals to Semliki forest virus. Although atabrine increases resistance of mice to equine encephalomyelitis virus(14), it exerts little or no influence on Semliki forest viral infection. With the exception of dichlorophenoxy thiouracil(4), isatin thiosemicarbazone(8) and atabrine(14), none of the materials mentioned has been demonstrated to protect mice when the virus is inoculated by the cerebral route.

Summary. 1. Feeding mice D-, L- or DL-ethionine in a casein-sucrose diet increases the survival rate of mice inoculated intraperitoneally with Semliki forest virus. A single, large dose of DL-ethionine injected intraperitoneally within 24 hours preceding viral inoculation by the same route also increases the survival rate. Ethionine does not increase the resistance of mice to Semliki forest virus when the virus is inoculated intracerebrally. Ethionine-treated mice which survive infection usually are immune to the virus. 2. Methionine does not counteract the protective action of ethionine against Semliki forest viral infection in mice. 3. Dichlorophenoxy thiouracil and closely related materials increase the survival rate of mice inoculated intraperitoneally with Semliki forest virus. 4. Benzimidazole, 2,6-diaminopurine and other purine derivatives tend to decrease the resistance of mice to Semliki forest virus.

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Received October 20, 1953. P.S.E.B.M., 1953, v84.

Copper, Molybendum and Zinc Interrelationships in Rats and Swine.* (20687)

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It is generally recognized that naturally occurring molybdenum in pasture plants of certain areas produces a severe disease of cattle and sheep which is apparently cured or prevented by supplemental copper. The literature on molybdenum in relation to the health of farm animals has been reviewed to 1944 by Russell(1) and the influence of molybdenum on the copper metabolism of animals has been reviewed to 1952 by Marston(2). Most of this experimental work has been done with rats, sheep and cattle. While chronic molybdenum toxicity symptoms are similar to those of copper deficiency, there does not seem to be a simple stoichiometric relationship between the two elements, and the existence of other variables is indicated. The role of molybdenum and copper in various flavin enzyme systems(3-5) may need to be considered in any explanation of toxic effects and antagonisms. Of particular interest are the findings that under some conditions molybdenum causes a depletion in copper reserves, whereas under others, there occurs a tissue accumulation of copper coincidental with symptoms of copper deficiency(2). There has been some indication of a relationship between zinc and copper in that some of the effects of high dietary zinc in rats were overcome by copper supplementation(6,7). However, this relationship has not been well established. This study shows the effects of high levels of molybdenum, zinc and copper on the tissue storage of these elements and also upon the distribution of radioactive copper. Rats and swine were used, the latter because there is little or no information in regard to copper-molybdenum relationships in this species.

Methods. Weanling rats from a highly inbred Wistar strain and 10-week-old Hampshire pigs were each lotted into 6 groups and maintained on a basal ration to which copper, molybdenum, zinc or combinations were added at levels as indicated in Tables I and II. Molybdenum was supplied as sodium molybdate, and copper and zinc as the sulfate. The swine basal ration consisted of 61.3% ground yellow corn, 30% soybean oil meal (solvent process), 7% alfalfa meal, 1% sodium chloride and 0.7% calcium carbonate. It was found to contain by analysis 8.9, 2.1 and 39.2 p.p.m. of copper, molybdenum and zinc respectively. One group of the rats received a ration identical to that fed the swine, and the second group received a synthetic ration previously described(8) to which the copper, molybdenum and zinc supplements were added. The growth response and tissue analyses of the rats for the two groups were similar, and for brevity only results on the natural ration will be discussed. A third short term study with old and young rats involved administration of a molybdenum solution by stomach tube for 8 consecutive days to determine the effect of such supplementation upon tissue uptake of radiocopper intramuscularly administered and these results are presented in the discussion.

Swine were maintained for 27 weeks and rats for 14 weeks on the experimental rations. They were then dosed orally with radiocopper and placed in metabolism units for 24 hours previous to sacrifice for tissue distribution studies and tissue analyses(9-11). Each rat received 320 micrograms of labeled copper with an initial activity of about 100 microcuries and the swine received 75-100 mg of copper as the chloride containing an initial

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* Published with the approval of the Directors of the Tenn. and Fla. Agric. Exp. Stations. Taken in part from a thesis submitted by R. Kulwich in partial fulfillment of degree of Doctor of Philosophy in the Graduate School of the University of Florida. The radioactive copper was obtained from the Oak Ridge National Laboratory on allocation from the U. S. Atomic Energy Commission.

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TABLE I. Effects of Dietary Levels upon Accumulation of Copper, Molybdenum and Zinc in Rat Tissues and upon Uptake of Copper 64.*

	Basal ration	Basal + 1000 p.p.m. Mo	Basal + 1000 p.p.m. Zn	Basal + 200 p.p.m. Cu	Basal + Mo and Cu	Basal + Zn and Cu
Wt gain, g/8 wk	115	38	105	119	40	107
Copper (p.p.m.)						
Liver	14	48	13	17	415	13
Kidney	20	152	21	21	131	24
Molybdenum (p.p.m.)						
Liver	2.1	33	2.6	1.6	36	2.2
Kidney	1.0	146	1.7	<1	57	<1
Femur	<1	19	1.7	<1	19	1.8
Zinc (p.p.m.)						
Liver	65	101	100	77	127	71
Kidney	97	99	96	100	102	91
Femur	149	120	177	144	129	229
Copper 64†						
Blood	29	186	10	5	36	7
Liver	49	164	61	79	164	94
Kidney	257	397	298	233	136	273
Spleen	19	66	12	49	38	9
Muscle	2.1	16.5	1.2	1.8	4.0	1.7
Skin	6.0	22.9	8.2	5.4	13.7	3.6
Red bone marrow	395	650	86	125	158	151

* Values represent averages of individual tissues from 3-5 animals except for kidneys which were pooled. Femurs calculated on fresh wt basis, all others on dry wt.

† Reported as % of ingested dose × 10^3/g of fresh tissue corrected to body wt of 275 g.

activity of 25 to 30 millicuries. Samples for radiocopper assay were prepared by wet-ashing in concentrated nitric acid and diluting to a volume of 10 ml(12). Samples with sufficient radioactivity were transferred to a 60 mm Petri dish and counted directly with a thin mica end-window Geiger counter. With samples containing low levels of Cu 64, a one milliliter aliquot was pipetted into a tared stainless steel planchet and evaporated to dryness under an infra-red lamp. The dried samples were then counted in a restricted atmosphere proportional counter. Self-absorption corrections were applied as determined from an empirical self-absorption curve. Samples which were to be analyzed for total copper, molybdenum or zinc in addition to radioactivity measurements, were treated after radioassay, according to the method of Lindner and Harley(13) to yield a completely oxidized sample suitable for the chemical determinations. The carbamate method(14) was used for copper determinations, using isoamyl alcohol as the solvent. The thiocyanate-stannous chloride method, essentially as described by Perrin(15) was used for molybdenum determinations. Zinc was determined by the dithizone method(16).

Results with rats. To conserve space only average values are given. While there was considerable variation between individuals, the differences observed were so large that there is little question as to significance. As noted in Table I, the addition of 200 p.p.m. of copper or 1000 p.p.m. of zinc had no apparent effect upon growth rate of the rats whereas the addition of 1000 p.p.m. of molybdenum resulted in a reduced growth rate. The supplement of 200 p.p.m. of copper did not alter the effects on growth of the high molybdenum intake. When the rats were drenched daily with molybdenum the growth was also retarded and the effect was greater in weanlings than in 24-week-old animals.

Analyses of livers, kidneys, and femurs of the rats indicated that supplementary zinc or copper resulted in little increase in the concentration of copper, zinc or molybdenum in tissues after 14 weeks on the ration. However, rats receiving supplementary molybdenum either with or without copper showed appreciably higher copper concentrations in the liver and kidney, ranging from a 3-fold to a 30-fold increase. High dietary molybdenum

TABLE II. Effects of Dietary Levels upon Accumulation of Copper, Molybdenum and Zinc in Swine Tissues and upon Uptake of Copper 64.[*]

	Basal ration	Basal + 1000 p.p.m. Mo	Basal + 1000 p.p.m. Zn	Basal + 200 p.p.m. Cu	Basal + Mo and Cu	Basal + Zn and Cu
Daily gain (lb)	1.1	.95	1.0	.98	.96	1.0
Feed/100 lb of gain (lb)	423	462	415	397	417	444
Copper (p.p.m.)						
Liver	29	149	27	60	239	32
Kidney	40	144	47	59	60	43
Spleen	6.6	25	3.1	4.4	6.9	4.8
Plasma	238	376	260	231	330	247
Molybdenum (p.p.m.)						
Liver	5.0	67	5.6	4.6	32	5.0
Kidney	4.4	75	3.7	3.2	25	4.9
Spleen	1.1	21	.9	.4	8.8	.5
Femur shaft	1.9	32	.2	1.5	22	1.1
Copper 64[†]						
Blood	14	19	10	10	10	10
Liver	66	137	48	42	40	46
Kidney	75	80	59	50	37	67
Spleen	4.9	7.7	3.1	2.3	2.9	2.9
Muscle	2.4	2.2	1.3	2.1	1.5	1.2
Skin	2.6	2.7	4.9	3.9	2.7	4.1
Red bone marrow	1.2	3.4	1.3	.4	2.9	1.3

[*] Values represent averages of individual tissues from 3 animals in each lot. Plasma calculated as μg/100 ml, all other analytical results as parts per million on a dry matter basis.

[†] Values reported as % of ingested dose $\times 10^5$/g fresh wt corrected to a body wt of 200 lb. The No. of animals per lot were as follows: 2, basal; 1, basal + Mo; 3, basal + Zn; 2, basal + Cu; 3, basal + Mo and Cu; 3, basal + Zn and Cu.

was also found to increase the molybdenum content of the liver, kidney and femur.

The high molybdenum rats on the purified rations, the individual values for which are omitted, showed a 3-fold increase in skin copper concentration at the basal copper level and an even greater, though less uniform increase at the high copper level. Skin samples were taken from the back without removing the hair. It is of interest that Hundley and Ing(17) reported a 4-fold increase in copper concentration of skin samples of pantothenic acid-deficient black rats which showed a graying of the hair coat. These authors suggested that gray hair production in pantothenic acid deficiency may involve interference with copper utilization. Singer and Davis(18) reported graying of hair coat in piebald rats on a copper deficient ration which was corrected either by additional dietary copper or calcium pantothenate. These findings support the viewpoint that the high copper concentrations in skins of rats showing graying of the hair coat, after being fed rations deficient in pantothenic acid but not in copper, may reflect the presence of physiologically unavailable copper. In the light of these findings it is plausible to assume that the high copper concentrations found in the skin and other tissues of rats maintained on high levels of dietary molybdenum may also represent copper that is physiologically unavailable.

Results obtained from the tissue distribution of radiocopper (Table I) provide further evidence of increased copper retention by animals fed supplementary molybdenum. At the basal copper level, uptake of copper 64 by blood, liver, kidney, spleen, muscle, skin and red bone marrow was increased in those rats receiving high levels of molybdenum, although there was considerable variability within groups. At 200 p.p.m. dietary copper, the high molybdenum groups showed increased radiocopper uptake by all tissues examined except kidney and spleen. The groups receiving the high level of dietary zinc at the basal copper level showed evidence of lowered blood and red bone marrow copper 64 accumulation, but this did not occur at the high level of copper intake.

Drenching with molybdenum for 8 consecutive days was likewise shown to increase the uptake of Cu 64 administered intramuscularly to 24-week-old rats. The blood showed a 3-fold increase; spleen, skin and liver showed smaller increases but the kidney value was lowered. A somewhat similar pattern was noted with weanling rats. Copper 64 uptake by the blood in the molybdenum groups was increased over 3 times that found in the control group. The skin and liver showed a slight increase in Cu 64 uptake for the molybdenum-drenched group, while kidney and spleen values were lower than those for controls.

Results with pigs. Growth rate and feed consumption data for the various lots of swine (Table II) indicated that the addition of 200 p.p.m. of copper or 1000 p.p.m. of molybdenum or zinc to a natural ration did not result in any marked growth retardation nor did it influence the feed utilization efficiency. Pigs fed 1000 p.p.m. of zinc in this experiment showed no ill effects although they received a lower level of zinc, on a dry matter basis, than did those in the experiments reported by Grimmett *et al.*(19), in which 1000 p.p.m. of zinc as the lactate was added to whole milk with pigs becoming lame and unthrifty within two months. Apparently zinc accumulation in the present experiment had not reached the toxic level.

Histological examination of sections taken from the cerebrum, cerebellum, cervical cord, thoracic cord and lumbar cord of pigs from all lots showed no degenerative changes and were believed to represent normal tissues. Leg joints and muscles taken from the gluteal region showed no pathological change.

Analytical data for copper, molybdenum and radiocopper are also presented in Table II. Ingestion of 200 p.p.m. of supplementary copper did not cause any increase in spleen or plasma copper levels when compared to the basal group, although kidney and liver values were somewhat higher. Supplementary zinc did not appear to affect copper accumulation in these tissues studied. Dietary supplementation with molybdenum, on the other hand, appeared to markedly increase tissue copper accumulation.

At the basal copper level, pigs receiving supplementary molybdenum showed a 5-fold increase in liver and about a 4-fold increase in spleen and kidney copper concentration. Plasma copper levels were elevated about 58%. An increase in tissue copper concentration by molybdenum supplementation was also found at the high copper level; there was a 4-fold increase in liver copper concentration and a 43% increase in plasma copper concentration observed in the control group. However, when high dietary copper levels were fed, supplementary molybdenum did not appear to materially affect kidney or spleen copper concentration.

Ingestion of 1000 p.p.m. of molybdenum resulted in high molybdenum values for liver, kidney, femur and spleen. Molybdenum concentration in these tissues from groups receiving supplementary copper in addition to molybdenum, however, were considerably lower than corresponding values for groups receiving only supplementary molybdenum. This indicates that the excess copper intake lowered the accumulation of dietary molybdenum. Accumulation of molybdenum was apparently unaffected by the high zinc level.

Unfortunately, copper 64 uptake data were obtained for only one pig of the lot receiving supplementary molybdenum at the basal copper level. The data for this animal indicated an increased concentration in liver, spleen and red bone marrow of pigs on the basal ration plus high molybdenum. At the high copper level, however, the lot receiving supplementary molybdenum showed a definite increase in copper 64 uptake only in the red bone marrow. High dietary level of zinc did not appear to have any appreciable effect upon the uptake of labeled copper.

The increased tissue copper accumulation observed in swine fed high dietary molybdenum levels is in agreement with the rat data and the results of Marston with rats who reported that supplementation with 1 mg of molybdenum daily resulted in increased copper accumulation.

Summary and conclusions. Substantial increases in tissue copper concentrations and radiocopper accumulation resulted when a level of 1000 p.p.m. of molybdenum as sodium molybdate was fed to rats and swine on a natural ration. This increase was especially pronounced in the kidneys and livers and is in accord with the hypothesis that excess copper, which is not physiologically effective, may be accumulated when a high level of molybdenum is fed. A dietary level of 1000 p.p.m. of zinc did not appear to have any appreciable effect upon copper metabolism. Growth depression in rats produced by 1000 p.p.m. of added molybdenum to a natural or synthetic ration was not counteracted by the addition of 200 p.p.m. dietary copper. Swine appeared to be more resistant to molybdenum toxicity than did rats. No degenerative changes were observed in the spinal cord, leg joints or muscle tissues of swine fed levels of 1000 p.p.m. of molybdenum or 1000 p.p.m. zinc for a 7-month experimental period. With the criteria of these experiments it appears that dietary zinc was not involved in copper-molybdenum imbalance.

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Received October 26, 1953. P.S.E.B.M., 1953, v84.

Fat Storage of an Estrogen in Women Following Orally Administered tri-*p*-anisylchloroethylene. (20688)

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It was reported that tri-*p*-anisylchloroethylene (TACE*) administered orally to ovariectomized rats and monkeys produced estrogenic activity of long duration(1,2). This prolonged action following oral administration resulted from storage of estogenic material in the body fat and its slow release(1,2). Greenblatt and Brown(3) demonstrated estrogenic material in the body fat of human beings given TACE and none in that from patients receiving other synthetic and natural estrogens. Since fat storage of estrogen is of therapeutic significance, further studies extending those of Greenblatt and Brown are reported.

Methods. Fat biopsy specimens† obtained

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† The writers are indebted to Drs. R. B. Greenblatt, Medical College of Georgia, Augusta, Ga.; K. J. Karnaky, Baylor College of Medicine; P. Abramson, Shreveport, La.; and Wm. Bickers, Richmond, Va., for obtaining the fat biopsy specimens.

TABLE I. Estrogenic Content of Fat Biopsy Specimens Obtained from 14 Women Administered TACE.

Dose/day (mg)	No. days treated	Time from last dose to biopsy	Estrogenic content of fat (mg % TACE equivalent)
48	1	22 hr	.18
192	1	15	0
36	3	18	Trace
48	7	12	.38
72	9	20	.42
72	14	1	.49
72	16	61	.42
24	104	5	.50
24	166	4½	.58
24 48	193 and 103	15	.93
48	111	3½	.80
96	27	4	4.41
24	166	23 days	.2-.4
96	27	34	.25
60	10	15	.50
72	16	15	.54

from omental or subcutaneous tissues were immediately placed in a mixture of equal parts benzene and ethyl alcohol. The tissue, in the solvent, was macerated in a Waring Blender and the homogenate was filtered through cheese cloth. The residue and the cheese cloth were washed twice on a funnel with the solvent; the filtrates were combined and evaporated to drynes over a steam bath. The estrogenic content of the oily residue was estimated by the uterine weight method of Evans, Hines, Varney and Koch(4). The oily residue was administered subcutaneously to the mice either undiluted or diluted with olive oil.

Results. Bioassay results are summarized in Table I. No estrogenic material or only trace amounts were present in the body fat of women who received oral TACE in doses of 48 mg and 192 mg in one day or 36 mg daily for 3 days. Biopsy specimens obtained from patients given 48 or 72 mg of TACE per day for 7-16 days or 24 mg per day for 104 and 166 days, contained estrogenic material equivalent to 0.38-0.58 mg % TACE. Estrogen concentrations of the fat were somewhat greater in 2 patients who received 48 mg for 103 and 111 days. The fat from one patient given twice this daily dose (96 mg) for a shorter period (27 days) contained a considerably greater amount of estrogenic material (4.41 mg % TACE equivalent). From the fifteenth to the thirty-fourth day following TACE withdrawal significant amounts of estrogenic material were recovered from the fat of 4 patients given various doses of TACE for 10 to 166 days. This study suggests that the estrogenic content of fat is dependent upon both the size of the dose and the number of days of TACE administration.

Clinically a single 30-day course of therapy with TACE has frequently controlled menopausal symptoms for prolonged periods(5) and there has been a low incidence of withdrawal bleeding(6). These observations can be related to the fat storage of an estrogenic substance which is slowly and uniformly released.

Summary. The repeated oral administration of TACE to human subjects results in storage of considerable amounts of estrogenic material in the body fat. This material remains in the fat for prolonged periods following cessation of TACE therapy. Results of studies in a limited number of cases suggest that both duration of therapy and the size of the doses are contributing factors governing the level of estrogenic material in the fat.

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Received September 24, 1953. P.S.E.B.M., 1953, v84.

Comparative Effects of Chemotherapeutic Agents on Brown-Pearce Tumor and Normal Rabbit Testis *in vitro*.* (20689)

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(Introduced by Alfred Gellhorn.)

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Evidence has recently been cited(1) that the effectiveness of the guanine analog, 8-azaguanine, as a tissue inhibitor is related to the rate at which the tissue is able to deaminate this compound to its completely inactive derivative 8-azaxanthine. Normal and neoplastic tissues with a high deaminase activity were shown not to be susceptible to 8-azaguanine, whereas tumors in which the deaminase activity was low were inhibited. Chromatographic tests of feeding fluids from human glioblastomas cultivated *in vitro* have indicated this further, these susceptible tumors being almost entirely lacking in deaminating capacity(2). By this method the feeding fluids from tissue cultures have provided a more sensitive test of deamination than tissue homogenates. Since the Brown-Pearce rabbit carcinoma is known to be susceptible to 8-azaguanine *in vivo*(3,4) and since homogenates of this tumor as well as of normal rabbit testis have a negligible deaminase activity(1), it seemed desirable to study the effect of this agent on these tissues *in vitro*. Chromatographic tests of the feeding fluids were made to determine the azaguanine-deaminating capacity of the tissues. For comparison, the effects of the structural analogue 8-azaxanthine, and of benzimidazole(5), and 1,4-dimethanesulfonyloxybutane(6), an unrelated aliphatic carcinostatic agent, were also investigated.

Material and methods. The Brown-Pearce tumors used for culture were carried in the anterior chamber of the rabbit's eye. They were grown *in vitro* by the Maximow double coverslip method using a feeding medium of 3 parts human placental serum, 1 part beef serum ultrafiltrate and 1 part chick embryo extract. The tissue was cultured for one week during which the feeding solution was changed 3 times and the explants patched with chicken plasma once. At the end of this period the experimental solutions were added to the feeding media in the desired concentrations. For each test approximately 50 explants from 3 different tumors were used. The testicular tissue used for cultivation was obtained from healthy 2-3-month-old rabbits. It was grown *in vitro* in the same manner as the tumor tissue and was cultured for one week before the addition of the desired agent. Approximately 50 explants from 3 different rabbits were used for each test. A 0.1 M stock solution of 8-azaguanine was prepared by heating the solid in one-third the final volume of 0.5 N NaOH until a clear solution was obtained. This was then adjusted to pH 8 with 1 N HCl and made up to volume. It was applied to the tumor cultures in final concentrations of 0.0003 M, 0.0008 M, and 0.0016 M, and to the testis cultures in final concentrations of 0.0008 M and 0.0016 M. A control solution containing only NaOH in the above concentration was included in each experiment. The 8-azaxanthine solution was prepared in the above manner and applied to the tumor in a final concentration of 0.0016 M. Benzimidazole was prepared in a similar manner using 0.1 N NaOH instead of 0.5 N NaOH and was employed in final concentrations of 0.0016 M and 0.005 M. An 0.1 M stock solution of 1,4-dimethanesulfonyloxybutane was prepared by dissolving the solid in one-third the desired volume of propylene glycol and diluting to volume. This solution was applied to the tumor in final concentrations of 0.01 M, 0.005 M and 0.0016 M. A control containing propylene glycol alone was used in this case.

For permanent record and detailed study the cultures were fixed in Helly's fluid and stained with Harris' haematoxylin at the conclusion of the experiments. For the chromato-

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* This investigation was supported by grants from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

TABLE I. Effects of Chemotherapeutic Agents on Brown-Pearce Tumor and Normal Testicle *In Vitro.*

	Day of occurrence		
	.0016 M 8-Azaguanine		.005 M Benzimidazole
Effect	Brown-Pearce tumor	Normal testicle	Brown-Pearce tumor
Increased cytoplasmic granulation	1–2	1–2	1–2
Nuclear membrane more distinct	—	—	1–2
Elongate cells clubbed	—	1–2	2–3
Clumping	1–2	—	—
Proliferation stopped	2–3	2–3	3–4
Binucleate or multinucleate cells formed	ca. 2 mostly binucleate	—	ca. 5 multinucleate
Migration slowed	ca. 2	—	4–5
Round-cell shrinkage, pyknosis	2–3	—	blebbing 3–4
Vacuolization	—	—	ca. 5
Nuclear disintegration	ca. 3	3	5–7
Cell disintegration	4–5	4–5	ca. 7
Migration stopped	5	5	9–10

graphic tests the tumor or testicular tissue was grown in roller tubes. Each tube contained 5 explants which were fed a medium consisting of 3 parts balanced saline, 3 parts human placental serum, 3 parts beef serum ultrafiltrate and 1 part chick embryo extract. The tissue was cultivated for one week after which 8-azaguanine was added to the feeding fluid so as to produce a final concentration of 0.005 M. A control containing NaOH alone was used. Collection of the feeding fluids was made after 24 hours. The determinations were made in the same manner as those of the human glioblastomas(2).

Results. The Brown-Pearce tumor proliferates fairly rapidly *in vitro* and is characterized by two types of cells—one of elongate fibrocytic appearance and the other round and of fairly uniform size. The cells are mononuclear for the most part and tend to grow in sheets from the explant. The morphological characteristics of this tumor in tissue culture have been more fully described by von Möllendorff(7) and by Favorite and Cheever(8).

Of the 4 compounds under investigation, 2 failed to exert any demonstrable effect on the Brown-Pearce tumor *in vitro*. 8-Azaxanthine, although applied to the tumor at a concentration comparable to a consistently lethal level of 8-azaguanine (0.0016 M), had no cytological or inhibitory effect during the 10-day exposure period. 1,4-Dimethanesulfonyloxybutane was inactive at concentrations of 0.01, 0.005, and 0.0016 M. Table I summarizes the results obtained with the 2 active agents. 8-Azaguanine in general caused the round cells to shrink and become more granular. The cells tended to stick in clumps instead of migrating in the normally diffuse manner. Inhibition of cell proliferation occurred, followed by an increase in binuclear forms, later by nuclear and cell disintegration and finally by total inhibition of cell migration. The rate at which these effects were exerted varied directly with the concentration—the 0.0016 M solution was lethal in 5 days and the 0.0008 in 10 days. The 0.0003 M solution failed to cause a complete inhibition of cell proliferation or migration within the 10-day test period. The number of binucleate cells also seemed to be correlated with the concentration—the 0.0016 M producing the most and 0.0003 M the least. In contrast to the round cells of the tumor, the fibrocytic type of cell was not affected at any of these concentrations.

Benzimidazole when applied in a 0.0016 M solution did not affect the tumor in any way, but at 0.005 M inhibited proliferation in 3 to 4 days and migration in 10 days. This lethal effect was accompanied by a marked increase in the number of multinucleate cells. The cells also were shrunken and had more distinct nuclear membranes and a more granular and vacuolar cytoplasm than did the controls, and they also developed blebs or blisters. These changes were accompanied by nuclear and finally by cellular disintegration. Benzimidazole also affected the *fibrocytic* type of cell, causing it to become more blunt and rounded than normal. In general, however, this agent was significantly less inhibitory than 8-azaguanine.

Normal testicular tissue proliferates rapidly *in vitro* forming large colonies of fibroblast-like cells among which occasional round spermatogonial cells are found. When treated with 8-azaguanine complete inhibition was obtained in 10 days with the 0.0008 M and in 5 days with the 0.0016 M. In both cases, the cells became more granular and less elongate and branched than normal. The nucleus tended to round up and become more conspicuous. The nucleoli were more darkly staining than those of the controls. This was followed by nuclear and cell disintegration. Spermatogonial cell formation was also completely inhibited.

The chromatographic determination of azaguanine deaminase activity of the Brown-Pearce tumor and normal testicle demonstrated that a very large portion of the 8-azaguanine added in the feeding fluids (85-88%) remained unchanged after a 24-hour incubation period, indicating that the deaminase activity of these two 8-azaguanine-susceptible tissues was very low.

Discussion. Two main points of interest emerge from these comparative experiments. The effectiveness of 8-azaguanine and the complete inactivity of 8-azaxanthine are entirely consistent with the pattern demonstrated in a variety of tumors *in vivo*(1) and in the human glioblastomas *in vitro*(2). The inhibitory effect of 8-azaguanine on tissue cultures of this tumor appears to be correlated with the inability of the tissue to deaminate the carcinostatic agent to the inactive derivative, 8-azaxanthine. The negligible azaguanine deaminase activity of both the tumor and normal testicle *in vitro*, demonstrated by paper chromatography, reinforces this correlation.

Tissue cultures of this rabbit tumor and of rabbit testis are much more susceptible to 8-azaguanine than those of the human glioblastomas studied in earlier experiments(2); comparable effects were obtained at concentrations of 0.0008 M in the first two tissues and 0.005 M in the latter.

A comparison of the effects of these 4 agents on the Brown-Pearce tumor *in vivo* and *in vitro* points up significant discrepancies, as well as similarities, between these biological systems. On the one hand, the action of 8-azaguanine and 8-azaxanthine on this tumor is the same in the anterior chamber of the eye and in tissue culture: the former is strongly inhibitory in both systems and the latter has no effect. On the other hand, there is a wide divergence between the results *in vivo* and *in vitro* in the experiments with the other two agents. Benzimidazole, which does not cause any appreciable inhibition of the Brown-Pearce tumor in anterior chamber transplants (9), inhibits the growth of this tumor in tissue culture; 1,4-dimethanesulfonyloxybutane, which has been shown to have an inhibitory action *in vivo*(10), fails to affect the tumor *in vitro* in any way, even at levels 12 times as great as the least effective concentration of 8-azaguanine. These discrepancies invite speculation, but no established facts can be adduced at the present time to explain them.

Summary. 1. The effects of 8-azaguanine, 8-azaxanthine, benzimidazole, and 1,4-dimethanesulfonyloxybutane on the Brown-Pearce tumor *in vitro* were investigated. 8-Azaguanine proved lethal in 0.0016 M concentration in 5 days and in 0.0008 M concentration in 10 days, and benzimidazole was lethal in 0.005 M concentration in 10 days. 8-Azaxanthine and 1,4-dimethanesulfonyloxybutane proved ineffective in all concentrations used. 2. The comparative effects of 8-azaguanine on the Brown-Pearce tumor, which does not exhibit azaguanine deaminase activity, and on the normal rabbit testis, which also lacks this deaminase, were studied; and in both instances complete inhibition was obtained in the same time and at the same concentration. Paper chromatography tests on the feeding fluids of these 2 tissues also showed them to have negligible deaminase activity. 3. No reason is yet known for the disagreement, in the case of this rabbit tumor, between the results *in vivo* and those *in vitro* as regards inhibition by benzimidazole and by 1,4-dimethanesulfonyloxybutane (Haddow's GT 41); for 8-azaguanine, however, the results are consistent in animal and tissue culture.

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Received October 6, 1953. P.S.E.B.M., 1953, v84.

Effect of Aliphatic Oxime and Isatin Thiosemicarbazones on Vaccinia Infection in the Mouse and in the Rabbit.* (20690)

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Mice inoculated intracerebrally with vaccinia virus and treated with certain thiosemicarbazones containing either benzene or heterocyclic groups are protected against the virus (1-3). The concentrations of virus in the brains of mice treated with isatin thiosemicarbazone (T.S.) and sacrificed 5 days after initiation of infection was found not to be significantly different from those present in untreated animals(2). Further investigation of the mode of action of thiosemicarbazones in vaccinia infection has demonstrated 1) that serial passage of the virus in treated mice results in a gradual decrease in the amount of virus demonstrable in the brain; 2) that isatin T. S. fails to protect rabbits against vaccinia virus inoculated by the cerebral route; and 3) that aliphatic oxime derivatives of thiosemicarbazone may possess the capacity to protect mice against vaccinia virus. Results obtained from these experiments will be presented in this report.

Methods and materials. The International Health Board (IHD) and the Williamsport strains of virus were used(1,2). Infected mouse brain (IHD strain) or rabbit testicular tissue (Williamsport strain) was ground in a mortar and diluted with Locke's solution containing 5% normal rabbit serum. Harlan Swiss female mice weighing from 15 to 20 g were anesthetized with nembutal and inoculated intracerebrally with appropriate viral dilutions. Rabbits weighing approximately 2 kg, each, were inoculated either intracerebrally or intracisternally. Test materials were fed in a casein-sucrose diet or injected intraperitoneally as a suspension in sterile distilled water.

Results. Two experiments were caried out to determine the effect of serial passage of the Williamsport strain of virus in mice treated intraperitoneally with isatin T.S. (Table I). Animals were treated on the day of viral inoculation and on 2 succeeding days and sacrificed on the 5th day. Brains were removed from 6 mice after each transfer; those found free of bacterial contaminants were pooled and appropriate dilutions prepared. Mice were inoculated for transfer purposes and the viral content of the pool determined by duplicate intradermal titrations in 2 rabbits.

In Exp. 1, mice were treated with 5 mg doses of the compound and passages were

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* This work was supported, in part, by a contract between the Office of Naval Research, Department of the Navy, and Indiana University.

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TABLE I. Effect of Isatin Thiosemicarbazone on Concentration of Vaccinia Virus in the Brain of the Mouse.

		Conc. (log) of virus in brains of mice sacrificed 5 days after intracer. inoculation of virus			
		Rabbit titration		Mouse titration	
Exp.	No. of passage	Treated	Untreated	Treated	Untreated
1*‡	1	5.1	5.8		
	2	4.3	6.3		
	3	3.4	6.5		
	4	2.8	7.3		
2*§	1	5.5			
	2	4.4			
	3	3.8			
	4	3.4			
	5	2.1			
	6	2.3			
	7	1.4			
3†‖	1	<2.8	<2.5	2.0	<1.5
		3.1	4.7	<1.7	2.9
		4.3	5.3	2.7	2.3
		4.7	6.4	2.1	>4.1
		5.1	6.8	3.1	3.7
		6.1	6.8	>4.0	4.3
Composite titer		4.3	5.2	2.4	3.4

* Brains from 6 mice sacrificed at each passage were pooled before titration. Passages were made with 10^{-3} dilution of brain tissues in Exp. 1 and 10^{-2} in Exp. 2.

† Individual brains were titrated.

‡ Williamsport strain; treatment 5 mg interper. 0,1,2 d.

§ Williamsport strain; treatment 2.5 mg intraper. 0,1,2 d.

‖ IHD strain; treatment 2 mg intraper. 0,1,2 d.

made with the 10^{-3} dilution. After 4 transfers, the viral titer had decreased 2.3 log units or an average of 0.77 log unit for each passage. In contrast, an increase in concentration of virus was observed in brains of untreated control mice. In Exp. 2, a dose of 2.5 mg of thiosemicarbazone was used and transfers were made with the 10^{-2} dilution of infected tissues. After 7 passages, the titer had decreased 4.1 log units or an average of 0.68 units for each transfer.

Since the possibility existed that treatment of mice with isatin T.S. might reduce virulence of vaccinia virus for the mouse but not for the rabbit, a third experiment was carried out in which brains of treated and untreated mice were removed 5 days after viral inoculation and titrated individually in rabbits and in mice. The IHD strain of virus was used in this experiment. The difference between the composite titers of the brains of 6 treated mice was 1.9 log units less when titrated in mice than when titrated in rabbits (Table I). A comparable value of 1.8 log units was obtained for 6 brains from untreated mice. The titer of untreated mouse brains was 0.9 log unit higher than for brains of treated animals when the tissues were titrated in rabbits and 1.0 unit higher when titrated in mice.

Tests in rabbits. In previous experiments it was observed that rabbits treated with thiosemicarbazones were not protected against vaccinia virus injected into the skin(3). Tests were made to determine whether protection could be obtained by similar treatment if the virus were inoculated by cisternal or cerebral routes. It was found that protection was not obtained when isatin T.S. was administered intraperitoneally in 3 doses of approximately 250 mg/kg, each, within 48 hours after viral inoculation.

In contrast, the fatality rate of mice inoculated intracerebally with the same dilutions of the same strain of virus was reduced by about 40% by similar treatment with doses of approximately 90 mg/kg of isatin T.S.

Aliphatic oxime thiosemicarbazones. Thiosemicarbazone derivatives of butanedione, containing oxime or methoxime groups, protect mice against vaccinia virus (Table II). The corresponding semicarbazones are inactive. When fed in a concentration of 0.1%, 1 - phenylethane - 1,2 - dione - 2-oxime-1-T.S. slightly increased the number of survivors in one experiment (Williamsport strain). Pyruvamide T.S. likewise exerts a protective action when fed in a strength of 0.2%. Other materials tested and found to produce little or no protection include acetylacetone di-T.S. (IU), octane - 2,3 - dione - 3 - oxime - 2 - T.S. (WRL), cyclohexan-1,2-dione-phenylhydrazone T.S. (WRL) and butane-2,3-dione-2-oxime-3-S-methyl thiosemicarbazone (WRL). When injected intraperitoneally in doses of 0.3 mg on 0, 1 and 2 days after viral inoculation, butane-2,3-dione-2-methoxime-3-T.S. increased the number of survivors (excess of 4). This compound as well as butane-2,3-dioneoxime T.S., 1-phenylethane-1,2-dione-2-oxime-1-T.S. and acetonylacetone di-T.S.,

TABLE II. Effect of Aliphatic Oxime Thiosemicarbazones and Related Materials on Vaccinal Infection in the Mouse (IHD Strain).

Source*	Compound	Conc. in diet (%)	Proportion of mice surviving infection†		Excess of survivors in treated groups
			Treated	Untreated	
EKC	Butane-2,3-dione oxime T.S.	.1	28/35	6/35	11‡
WRL	Butane-2,3-dione oxime semicarbazone	.1	4/17	4/18	0
"	Butane-2,3-dione, 2-methoxime, 3-T.S.	.015 .03	32/36 34/54	13/36 7/53	9‡ 9‡
"	Butane-2,3-dione, 2-methoxime, 3-semicarbazone	.1	5/18	4/18	1
"	Propane-1,2-dione,1-oxime, 2-T.S.	.1	9/18	5/18	4
"	Octane-2,3-dione, 3-oxime, 2-T.S.	.16	0/18	5/18	–5
"	1-Phenylpropane-1,2-dione-2-oxime-1-T.S.	.16	9/18	4/17	5
"	1-Phenylpropane-1,2-dione-2-methoxime-1-T.S.	.06	8/16	4/18	4
IU	Ethyl pyruvate T.S.	.2	6/18	4/18	2
"	Pyruvamide T.S.	.2	10/16	4/18	6
"	Acetylacetone di-T.S.	.1	1/18	5/18	–4

* Source of compounds: EKC—Dist. Products Industries; WRL—Dr. G. H. Hitchings, Wellcome Research Lab.; IU—Dr. E. Campaigne, Indiana University.

† Approx. equal numbers of mice (6 per group) received 10^{-2}, 10^{-3} and 10^{-4} dilutions of virus intracer.

‡ Composite of 2 or more experiments; avg No. of excess survivors per experiment listed.

in concentrations of 0.001 mg/ml, reduced viral multiplication to a variable degree in tissue cultures containing mouse embryonic tissues.

Minerals. Since butane-2,3-dioneoxime T.S. has been reported to be a chelating agent for manganese (4), tests were made to determine whether this element would counteract the protective capacity of thiosemicarbazones against vaccinia virus. In these experiments, butane-2,3-dioneoxime T.S., butane-2,3-dione-2-methoxime-3-T.S. and isatin T.S. were used. Although, in some experiments, fewer mice survived in groups fed a combination of manganese chloride (.5-1.0%) and thiosemicarbazone than in those treated with thiosemicarbazone alone, the results were irregular and are not interpreted as a reversal of the protective capacity of the thiosemicarbazone. No reduction in the number of survivors was obtained by feeding thiosemicarbazones in combination with copper chloride (0.05%), cobalt nitrate (0.05%), zinc chloride (0.1%) or sodium molybdate (0.015%). Chelating agents, including dimethyl glyoxime, 8-hydroxyquinoline, ethyl xanthate and the sodium salt of tetraacetic acid, also were tested but were found not to influence the resistance of mice to vaccinia virus.

Discussion. The data which have been presented do not explain the mode of action of thiosemicarbazones in vaccinia infection. Isatin T.S. protects mice against virus inoculated intracerebrally but fails to protect rabbits. In the mouse, the concentration of virus which develops in the brain following intracerebral inoculation is somewhat less in treated than in untreated animals. Nevertheless, it would appear that the concentration of virus in the brains of treated mice may reach a level which would not be tolerated by untreated animals. Isatin T.S. must exert either a direct or indirect action on the virus, however, since maintenance of the virus in treated mice results in a gradual diminution in the amount of virus demonstrable in the brain.

It has been demonstrated that a cyclic component is not essential for antiviral activity of thiosemicarbazones. Although the molecular structure of aliphatic oxime derivatives possessing protective capacity would indicate that such compounds could function as chelating agents, evidence has not been obtained that these or other thiosemicarbazones act by interfering with mineral metabolism nor that altered mineral metabolism influences resistance of mice to vaccinia virus. The activity of the methoximes, in which metal chelation would be blocked, also points in this direction, although the possibility of *in vivo* demethylation cannot be ruled out. While it is possible to pick out a structural unit, $=N-C-C=NNHCSNH_2$, which is common to most of the active derivatives, a few active thiosemicarbazones outside the scope of this unit have been found, *e.g.*, benzaldehyde T.S., and a few compounds which possess this unit are inactive, *e.g.*, cyclohexanedione phenylhydrazone T.S. Modifications of the sulfur as in the semicarbazones and S-methyl derivatives result in complete inactivation.

The antibiotic, Netropsin, resembles the thiosemicarbazones in that it protects mice inoculated intracerebrally with vaccinia virus but fails to prevent the development of lesions at the site of virus inoculated into the skin of rabbits(5).

Summary. 1. Serial passage of vaccinia virus intracerebrally in mice treated with isatin thiosemicarbazone results in a gradual diminution in the amount of virus demonstrable in the brain. 2. Treatment with isatin thiosemicarbazone fails to protect rabbits inoculated intracerebrally with vaccinia virus. 3. Aliphatic oxime thiosemicarbazones may be as effective as benzene or heterocyclic derivatives as chemoprophylactic agents against vaccinia virus in mice.

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Received October 20, 1953. P.S.E.B.M., 1953, v84.

Effect of Diet on Rat Liver Betaine Transmethylase.* (20691)

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The methylation of homocysteine by betaine in the presence of rat liver preparations *in vitro* was first demonstrated by Borsook and Dubnoff(1). The activity of this enzyme system, called betaine transmethylase, has been shown to be reduced when animals are subjected to a deficiency of folic acid(2) or vit. B_{12}(3). The present experiments were designed to investigate further the effects of diet on the enzyme system.

Methods. Weanling Sprague-Dawley rats were used. The basal diet contained alpha protein,† 18 g; cod liver oil, 3 g; Crisco (hydrogenated vegetable fat), 10 g; salt mix (4), 2 g; sucrose, 66.4 g; inositol, 0.1 g; choline chloride, 0.1 g; DL-methionine, 0.43 g; α-tocopherol acetate, 25 mg; thiamine hydrochloride, 0.5 mg; riboflavin, 0.5 mg; folic acid, 0.5 mg; pyridoxine, 0.5 mg; nicotinic acid, 2 mg; calcium pantothenate, 1 mg; 2-methyl-1,4-naphthoquinone, 25 γ; biotin, 5 γ; vitamin B_{12}, 5 γ. The alpha protein was washed exhaustively with hot water, 95% ethyl alcohol, then dried and ground before incorporation into the diets. This basal diet was fed without supplement to some animals.

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* Research paper No. 990, Journal Series, University of Arkansas. This work was supported by grants-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, and from the National Institutes of Health, Public Health Service.

† Alpha protein is an isolated soy bean protein obtained from the Glidden Company, Chicago, Ill.

TABLE I. Methionine Formation by Livers of Rats Fed Various Diets.

Diet	Series	No. rats	Avg daily wt gain, g	Avg methionine formed, γ
C*	1	8	—	715 ± 113†
B	"	6	—	3 ± 3
C	2	6	4.1 ± .4†	662 ± 117
B	"	6	1.4 ± .1	36 ± 40
B + 10% casein	"	6	2.2 ± .1	646 ± 232
B + complete amino acid mix	"	4	2.2 ± .2	553 ± 153
B + liver extract‡	"	4	2.1 ± .4	65 ± 39
B + 1% yeast extract§	"	4	2.7 ± .3	68 ± 45
B + valine, leucine, isoleucine	3	2	—	0
B + cystine, threonine, phenylalanine, tryptophan	"	2	—	332 ± 172
B + lysine, histidine, arginine, methionine	"	2	—	33 ± 33
B	4	4	1.9 ± .3	28 ± 28
B + complete amino acid mix	"	4	2.3 ± .3	1056 ± 146
B + cystine, threonine, phenylalanine, tryptophan	"	5	3.2 ± .1	406 ± 94
B + cystine	"	6	1.9 ± .3	120 ± 89
B + threonine	"	4	3.9 ± .2	188 ± 37
B + phenylalanine	"	4	1.8 ± .2	48 ± 31
B + tryptophan	"	4	1.0 ± .1	620 ± 278

* C = Chow; B = Basal.

† Stand. error.

‡ 1:20 extract of liver, Nutritional Biochemicals, Cleveland, Ohio.

§ Yeast extract powder, Nutritional Biochemicals.

Other groups received the basal diet with a supplement of the essential amino acids and cystine. This amino acid supplement, referred to as complete amino acid mix in Table I, consisted of the following expressed as g per 100 g of diet: DL-valine, 1.74; DL-leucine, 2.09; DL-isoleucine, 1.39; L-cystine, 0.26; DL-threonine, 1.22; DL-phenylalanine, 1.04; L-tryptophan, 0.35; L-lysine monohydrochloride, 1.30; DL-histidine, 0.83; L-arginine monohydrochloride, 0.43; DL-methionine, 0.52. Other groups of animals were given the basal diet with various combinations of the above amino acids and with single amino acid supplements. In all these diets the amino acids were added to the diet at the same level at which they were added in the complete amino acid mix. Control animals were given a diet of a commercial laboratory chow.

The diets were fed for 2 weeks, then the animals were killed and the liver homogenates assayed for betaine transmethylase activity by the method previously described(2). Final substrate concentrations were: DL-homocysteine monohydrochloride, 64 mg %; and betaine, 128 mg %. The results are reported as μg of methionine formed per g of liver during the 3-hour incubation period.

The *results* presented in Table I show that livers from rats fed the basal diet exhibited no demonstrable betaine transmethylase activity. Supplementation of this diet with 10% casein or with the complete amino acid mix resulted in betaine transmethylase activities comparable to chow-fed rats. In contrast, yeast extract or liver extract was ineffective in restoring the enzyme activity. These results then indicated that the absence of betaine transmethylase in livers of the rats receiving the basal diet was due to an inadequate supply of amino acids. The remaining experiments were designed to determine which amino acids of the complete amino acid mix were active in producing the enzyme. The amino acids in the complete mix were arbitrarily divided into 3 groups and fed to rats. Only the combination containing cystine, threonine, phenylalanine, and tryptophan was effective. Livers from rats receiving these amino acids exhibited considerable betaine transmethylase activity but not as high an enzyme titer as was observed in livers from animals receiving the complete amino acid mix.

This observation was repeated in series 4 and in addition each of the 4 amino acids was added separately to the basal diet. Of the single amino acid supplements tested only tryptophan produced an appreciable betaine transmethylase activity.

The growth data given in Table I are of considerable interest. The basal diet permitted approximately half-normal growth. Supplementation of the basal diet with 10% casein, complete amino acid mix, yeast, or liver extract improved the growth rate. Supplementation of the basal diet with threonine resulted in growth equal to that obtained with rats fed chow. With respect to growth, methionine is the first limiting amino acid in alpha protein(5) and the present experiments indicate that threonine is the second limiting amino acid.

It is apparent from the data in Table I that there is no correlation between the effects of the various supplements on growth and on betaine transmethylase activity. Thus tryptophan supplementation resulted in considerable enzyme activity but actually depressed growth. Yeast extract improved growth considerably but was ineffective in stimulating the synthesis of the betaine transmethylase enzyme.

These observations suggest that the amino acid pattern required for the synthesis of this particular liver enzyme must be considerably different from the pattern required for growth of the animal. Also, the observation that some of the diets permitted good growth but allowed negligible synthesis of betaine transmethylase suggests that when rats are fed methionine the enzyme is not vital to the animal's economy. Although the observation periods in the present experiments were of short duration, in other experiments rats have been fed the basal diet for 5 months and continued to grow at a rate of approximately 2 g per day throughout this period.

Summary. 1. Rat liver betaine transmethylase varies strikingly with the amino acid composition of the diet. 2. The pattern of amino acids required for synthesis of this enzyme is considerably different from the pattern required for growth of the animal. 3. When rats are given methionine, betaine transmethylase does not appear to be necessary for growth.

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Received October 26, 1953. P.S.E.B.M., 1953, v84.

Effect of Antigonadotrophic Serum on Testes of A-Strain Mice Treated with Estrogen.* (20692)

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The development of interstitial cell tumors in A-strain mice after prolonged estrogen treatment has been demonstrated repeatedly(1-4). Gardner(5) has suggested that these tumors arise as the result of enhanced secretion of interstitial cell stimulating hormone (ICSH) by the pituitary gland under the influence of low doses of estrogen rather than from a direct effect of estrogen upon the testis. The present report presents the results of an attempt to test this hypothesis by nullifying the presumed excessive ICSH secretion with antigonadotrophic serum.

Materials and method. For the production

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* Aided by grants from the Anna Fuller Fund, the Damon Runyan Memorial Fund for Cancer Research, Inc., and the Society of Sigma Xi.

of the antiserum, a crude sheep pituitary extract[†] prepared according to the method of McShan and Meyer(6) was used. Adult rabbits were injected with amounts of the extract equivalent to 250 or 500 mg of acetone dried powder. This quantity is sufficient to increase the ovarian weights of immature female rats from the normal of 10-15 mg to 60-80 mg when assayed according to the method described by McShan and Meyer(6) and used by Deutsch *et al.*(8). Injections were given subcutaneously daily 5 times per week for 8 weeks, after which a series of 5 injections was carried out every third week only. At the end of the eighth week and at irregular intervals thereafter, the rabbits were bled from the central artery of the ear or by heart puncture. After cooling, the serum was recovered by centrifugation and stored in the frozen state until used. All serum assays for antigonadotrophic activity followed a method essentially similar to that described by Deutsch *et al.*(8). Only sera with demonstrable antigonadotrophic activity were used.[‡] *Male A-strain* mice 30-40 days of age were employed. These were penbred animals, the original stock of which was obtained from Dr. W. U. Gardner of Yale University. Injections of 16.5 μg of estradiol benzoate[§] in .05 ml of sesame oil were given subcutaneously weekly for 22 weeks. It was anticipated that within this interval the interstitial cell changes characteristic of estrogen treated animals would have become apparent.

At the end of this period, animals were divided into 4 experimental categories which continued to receive estrogen as described above. In addition to the estrogen, 5 daily subcutaneous injections per week of antigonadotrophic serum, normal rabbit serum, or pituitary extract were administered. *Normal rabbit* serum was chosen as the material which would most nearly fulfill the requirements of a control substance for the antigonadotrophic serum; inclusion of the series receiving pituitary extract was intended to provide a means of comparing the effect of an actively developed resistance with that of the passive resistance conferred by the rabbit antigonadotrophic serum.

The 4 groups of animals were treated as follows: Group 1 received .2 ml of antigonadotrophic serum, Group 2, .2 ml of normal rabbit serum, Group 3, an amount of pituitary extract equivalent to 25 mg of acetone dried pituitary powder in .25 ml of saline, and Group 4 received no treatment additional to the estrogen. A *fifth group* was made up of animals sacrificed at the outset of the experiment after 15 to 22 injections of estradiol, thus providing a means of evaluating the degree of interstitial cell hypertrophy and hyperplasia at the time injection of additional materials was initiated. *Injections* were continued until the animals became moribund, at which time autopsies were performed. The period of treatment extended from 3 injections of estradiol beyond the initial 22 injections in conjunction with 15 injections of either normal serum, antiserum, or pituitary extract to 10 injections of estradiol with approximately 50 injections of either of the added materials. Most animals, however, survived from 5-7 injections of estradiol in addition to 25-35 injections of the sera or extract (Table I). At *autopsy*, testes, seminal vesicles, and spleen were weighed and fixed in Bouin's solution, and in representative cases the adrenal, thyroid, and pituitary glands were similarly preserved. The testes were serially sectioned and lengths chosen at regular intervals along the ribbon for mounting. Either the hematoxylin and eosin or the periodic acid-Schiff method was used for staining. Approximate estimates of the degree of hypertrophy and hyperplasia were made by tracing selected sections at magnification of 60 X. The modified areas were measured with a planimeter and the total volume of the hypertrophied and hyperplastic cells was calculated for each

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[†] The author is indebted to Dr. W. H. McShan of the Department of Zoology, University of Wisconsin, for donating a portion of the pituitary extract and for generous assistance with the preparation of the remainder. The extract contains negligible amounts of lactogenic activity, but measurable thyrotrophic activity. On the basis of assays of short duration there appears to be little adrenotrophic or growth hormone activity(7).

[‡] Sera prepared from extracts similar to those employed in this study inhibited endogenous gonadotrophins in rats under varied experimental conditions (9-11).

[§] The estradiol benzoate was supplied through the courtesy of Dr. C. H. Sullivan of Ciba Pharmaceutical Products, Inc.

TABLE I. Effect of Antigonadotrophic Serum on the Development of Interstitial Cell Hypertrophy and Hyperplasia.

							Interstitial cell volumes			
Group	Treatment* (No. inj.)	No. animals†	Body wt (g)	Spleen wt (mg)	Testis wt (mg)	No. testes sectioned	% >.1 mm³	Mean (mm³)	% <.1 mm³	Mean (mm³)
1	Estrogen 3-10, antiserum 15-48	45	24.6 ± .71‡	321.6 ± 16.3	31.3 ± 1.78	53	24	1.65 ± .75	76	<.1§
2	Estrogen 3-13, Normal serum 15-59	33	24.5 ± .85	366.5 ± 23.3	77.4 ± 3.38‖	36	86‖	2.10 ± .56¶	14	
3	Estrogen 3-10, pituitary extr. 13-46	34	23.9 ± .62	248.6 ± 16.8	64.7 ± 4.18‖	42	59‖	1.00 ± .32¶	41	
4	Estrogen 3-10	27	26.6 ± .52	234.3 ± 15.5	99.1 ± 5.21‖	46	50‖	1.73 ± .70¶	50	
5	**	23	22.5 ± .64	160.3 ± 14.9	97.4 ± 3.14‖	25	32¶	.51 ± .21¶	68	

* Treatment begun after 22 inj. of estradiol. † Some animals included which had been dead several hr before autopsy. ‡ $SE = \frac{SD}{\sqrt{N}}$.

§ Majority of cases showed either no evidence of stimulation or too small to measure. ‖ $t = >2.5$, $p = <.02$. ¶ $t = <2.5$, $p = >.05$.

** Autopsied after 15 to 23 inj. of estradiol.

testis. Since the testes of an individual do not necessarily respond to the same extent, each testis was treated separately in measuring the degree of interstitial response. The total number of testes measured and the per cent of the total number showing volumes greater and less than .1 mm³ are given in Table I.

Results. The testes of the animals receiving estrogen and antiserum were greatly reduced in weight. Typical examples had small closely packed tubules. The internal organization of the tubules was destroyed. There were no mature sperm. Often the tubular content was limited to Sertoli cells, but a few scattered spermatogonia and spermatocytes were present as a rule. The extensive areas of hypertrophied and hyperplastic tissue typical of estrogen treated animals were totally lacking or represented by clusters of atrophied interstitial cells (Fig. 2, 5). Only 24% of the testes in this group had interstitial cell volumes greater than .1 mm³, whereas in the remaining 3 groups of treated animals 50 to 86% of the cases fell within this volume range.

The injection of normal serum or pituitary extract caused a reduction in testicular weight below that of animals which were given estrogen alone, but the effect was not as marked as in the case of antiserum treatment. Tubular atrophy in these groups was not as advanced as in the antiserum treated group, but more so than in the group receiving estrogen only. Tubules with mature sperm and intermediate stages of the spermatogenic series were frequently encountered. As indicated above, areas of interstitial hyperplasia and hypertrophy were numerous and extensive.

Sections from a normal serum treated specimen are shown in Fig. 1 and 4. A large area of hypertophied and hyperplastic interstitial cells is present. Tubular damage, as is usual in the vicinity of areas of extensive hypertrophy and hyperplasia, is well advanced. An example taken from Group 4, which received continued injections of estrogen only, is shown in Fig. 3 and 6. Many essentially normal tubules are seen in this testis. A small area of modified interstitial cells may be seen at the right.

The cytological changes characteristic of certain strains of mice when treated with

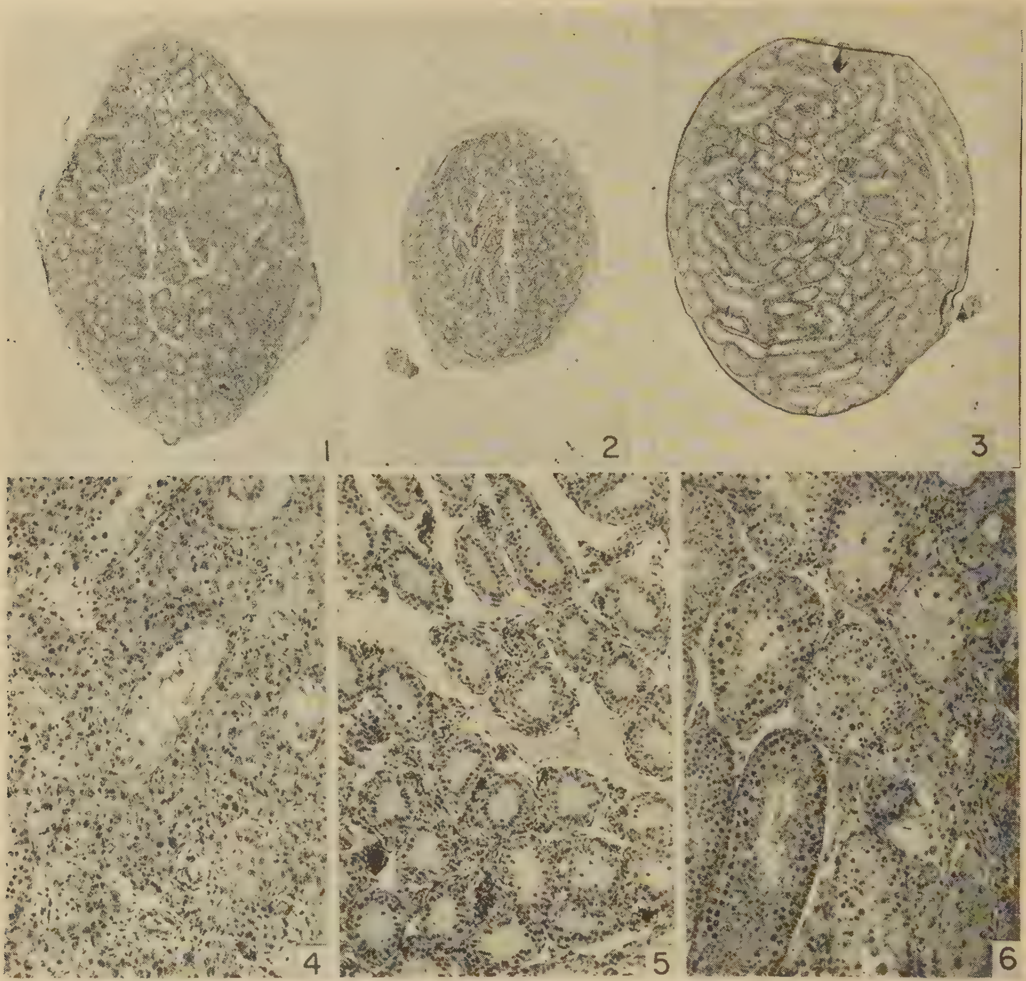

FIG. 1. Testis after 5 weekly injections of estradiol benzoate and 25 daily injections of normal rabbit serum in addition to 22 preliminary injections of estradiol benzoate. × 14. Total vol. of pretumorous area was 4.32 mm³.

FIG. 2. Testis after 8 weekly injections of estradiol benzoate and 37 injections of antiserum in addition to 22 preliminary injections of estradiol benzoate. × 14. There were no pretumorous areas of interstitial cells in this testis.

FIG. 3. Testis after 5 weekly injections of estradiol benzoate in addition to the 22 preliminary injections of the same material. × 14. Total vol. of pretumorous area 0.22 mm³.

FIG. 4. Same as Fig. 1. × 67.
FIG. 5. " " Fig. 2. " .
FIG. 6. " " Fig. 3. " .

estrogen have been described frequently(1-4). No striking departure from these observations was noted when modified interstitial cell areas in the testes from all experimental groups were studied.

The small series of adrenal, thyroid, and pituitary glands examined without recourse to quantitative procedures failed to show any marked differences between the group treated with normal serum and that treated with antiserum.

The body and spleen weights of groups receiving comparable treatment were not found to be significantly different.

Discussion. Until the comparatively recent work of Biskind and Biskind(12), Moon *et al.* (13,14) and of Griffin, Rinfert, and Corsigilia (15), there has been little experimental evidence to show that the secretions of the pituitary gland play an important role in tumor induction and growth. However, data accumulating from work in these laboratories has demonstrated that the role of the pituitary gland in tumorigenesis may be an important one. It is believed that the data presented here may be interpreted as further evidence of the participation of the pituitary gland in the development of neoplasms, inasmuch as it appears to support the hypothesis that the pituitary gonadotrophin, ICSH, is responsible for the induction of interstitial cell tumors.

The alternative possibility that the apparent suppression of early interstitial cell changes by antigonadotrophic serum is an aspecific effect is not to be overlooked. The lack of significant differences in spleen and body weights between the two groups of animals treated with normal rabbit serum and antiserum has been taken to indicate similarity of response to the rigors of the experimental procedure. However, further investigation into this aspect of the problem is needed and is to be carried out in connection with a study of the efficacy of antigonadotrophic serum when applied to fully developed interstitial cell tumors.

Summary. 1. Male A-strain mice treated simultaneously with estrogen and normal rabbit serum developed extensive interstitial cell hypertrophy and hyperplasia. Animals receiving an antigonadotrophic serum prepared by injecting a crude sheep pituitary extract into rabbits failed to show the same intensive interstitial cell changes. 2. These results have been interpreted as indicating that the effect of the excessive ICSH, presumed to be elicited from the pituitary gland under the influence of estrogen, has been nullified by the antigonadotrophic serum. The consequent suppression of the interstitial cell hypertrophy and hyperplasia, which normally occurs in this strain of mice when treated with estrogen, provides further evidence that the pituitary gland participates in growth and development of interstitial cell tumors. The possibility that the antiserum exerts an aspecific toxic effect is considered.

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Received October 29, 1953. P.S.E.B.M., 1953, v84.

Estimation of Estrogenic Activity of Genistein Obtained from Soybean Meal. (20693)

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The association of infertility of sheep in Australia with the estrogenic properties of subterranean clover(1,2,6,8) has stimulated interest in compounds of plant origin showing estrogenic activity. Bradbury and White(4) identified the isoflavone genistein, 4′, 5, 7 trihydroxyisoflavone, as the major constituent of subterranean clover responsible for its estrogenic activity. Their data also indicated that the chemical properties of the genistein isolated from the chloroplast fraction of subterranean clover were similar to those of the genistein isolated by Walz(10) and Walter(9) from soybeans. Walter(9) reported that dry hexane extracted soybean flakes contained 0.10% of the glucoside of genistein (genistin), an amount 50 times greater than was reported (4) to be present in fresh subterranean clover.

These quantitative considerations gave rise to the idea that soybean meal could be used as a source of genistein for biochemical investigations providing the genistein isolated was biologically active. Preliminary tests in the present investigation indicated that genistein isolated from soybean meal possessed estrogenic activity. The work reported herein deals primarily with a quantitative bioassay of the estrogenic activity of genistein isolated from soybean meal.

Experimental. Genistin was extracted from soybean meal,[†] and hydrolysed to its aglucone, genistein, according to the method of Walter(9). The test doses of the crystallized aglucone were dissolved in a minimum volume of ethanol and transferred to peanut oil[‡] in a 25-ml Erlenmeyer flask. The ethanol was removed under vacuum leaving the genistein dissolved in the peanut oil. (The genistein isolated had a melting point of 291-294°C and a specific absorption coefficient of 133 at 2625 Å). The bioassay of the estrogenic activity of genistein was conducted with immature female mice[§] (3 weeks of age) employing uterine weight response as the criterion. Diethylstilbestrol[‖] was used as the reference standard preparation. The test material as well as the diethylstilbestrol standard were injected subcutaneously twice daily for a period of 3 successive days. Twenty-four hours after the last injection the animals were sacrificed, the uteri removed, pressed lightly on a filter paper to eliminate uterine fluid and weighed. In preliminary tests the uterine-weight response curve of either the standard or the genistein preparation exhibited linearity over a wider range when plotted against the log of the dose rather than the arithmetical progression. Dose levels used in the subsequent assay were selected from the linear portion of the preliminary response curves. The assay was arranged as a 2-dose factorial(3) with 6 replications. The doses of genistein injected were 3 mg and 6 mg, and of diethylstilbestrol 0.015 μg and 0.030 μg. Each dose was dissolved in 0.6 ml of peanut oil and injected in 6 0.1-ml portions (representing 0.2 ml of oil per mouse per day). Controls were injected with oil alone.

Results. The results of the assay are presented in Table I. The mean uterine weight of the negative controls was 8.2 mg as compared with 23.8 mg for the genistein injected

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* Contribution from the Animal Industry Department, N. Carolina Agricultural Experiment Station, Raleigh, North Carolina. Published with the approval of the Director of Research as Paper No. 520 of the Journal Series. This investigation was conducted as part of a cooperative project with the Tennessee Valley Authority, Knoxville, Tenn. The authors wish to give acknowledgement to Dr. Paul E. Johnson for the advice and encouragement given by him to initiate this study.

[†] Solvent Extracted Soybean Meal.

[‡] Charlotte's Peanut Oil, Charlotte Refining Co., Charlotte, N. C.

[§] Mice furnished by M. P. Bailey of the North Carolina State Laboratory of Hygiene.

[‖] Diethylstilbestrol obtained from Jensen-Salsbery Laboratories, Inc., Kansas City, Mo. (Oil solution, each cc containing 2 mg)

TABLE I. Estrogenic Activity of Substances Tested. 6 mice in each series.

Treatments	No. mice	Mean uterine wt, mg
Injected		
Neg. control	6	8.2
Diethylstilbestrol		
.015 μg	6	20.3
.030 μg	6	32.4
Genistein		
3 mg	6	15.5
6 mg	6	32.2
Feeding		
Soybean meal with genistin extracted	5	8.1
Commercial soybean meal (solvent process)	6	23.0

TABLE II. Analysis of Variance of Two-Dose Factorial Assay.

	d.f.	S.S.	M.S.
Total	23	1876.53	
Replications	5	273.06	54.61*
Treatments	3	1311.45	437.15†
Difference between standard and genistein	1	36.75	36.75*
Combined slope of dose effect curve	1	1242.72	1242.72†
Divergence in slope	1	31.97	31.97*
Error	15	565.08	37.67

* Not significant ($P > .05$).
† Highly significant ($P \leq .01$).

mice. The slope of the response curve of diethylstilbestrol was not different from that of genistein over the range tested indicating that the biological action of the 2 preparations was similar over the range tested (Table II). The estrogenic potency of genistein computed from the data in Table I was 4.44 x 10^{-6} times the potency of diethylstilbestrol. Confidence limits (95%) placed on this estimate of potency gave a lower limit of 3.48 x 10^{-6} and an upper limit of 5.56 x 10^{-6}.

Discussion. The mean estimated potency of genistein obtained in this experiment seems to be different from that of genistein isolated from subterranean clover(4) (10^{-5} times as potent as estrone). This apparent difference probably is due to the fact that the estrogenic activity of estrone and diethylstilbestrol is different. Diethylstilbestrol has been reported(7) to be 16 times more effective than estrone when injected subcutaneously.

Cheng *et al.*(5) estimated the potency of genistein obtained from soybeans as 2 x 10^{-5} times that of diethylstilbestrol. This estimate of potency is approximately 4.5 times the potency obtained in this experiment. The difference apparently is not due to a diffqerence in uterine response to genistein but to the fact that Cheng(5) obtained a lower uterine response per unit of diethylstilbestrol than in the assay reported here.

In preliminary studies using the feeding method of bioassay, soybean meal from which genistein had been extracted was estrogenically inactive, whereas unextracted soybean meal was active (Table I). These observations indicate that the method of extraction was effective and point up the possibility of studying the biological effects of genistein with the unextracted meal.

On the basis of these current findings, nutritional studies are now in progress to determine the effects of estrogenic substances of plant origin on growth and reproduction.

Summary. Genistein of soybean origin was found to be 4.44 x 10^{-6} times as potent in estrogenic activity as diethylstilbestrol when measured by uterine weight responses of injected immature female mice. Commercial solvent extracted soybean meal also was found to be estrogenically active when fed.

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Received October 30, 1953. P.S.E.B.M., 1953, v84.

Reduction of Hyperkalemia by Circulating Blood through a Cation Exchange Resin.* (20694)

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(Introduced by William Dock.)

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Among the procedures devised to correct potassium intoxication in anuria, the use of the artificial kidney has proven the most effective (1). The complex equipment and elaborate preparation required for hemodialysis, however, limit its general availability. Removal of potassium from the gastro-intestinal tract of anuric individuals by the oral administration of a suitable cation exchange resin has been reported to be effective in lowering extracellular potassium concentrations(2). This method is not entirely satisfactory because of delays in effect and the occurrence of adverse gastrointestinal reactions. The following report describes preliminary observations on the correction of hyperkalemia after bilateral nephrectomy in dogs by circulating the blood through a cation exchange resin.

Method. The procedure was performed on 6 mongrel dogs 72 to 96 hours after bilateral nephrectomy. Each dog was anesthetized with pentobarbital sodium (25 mg/kg). A femoral artery and vein were exposed, a No. 16 needle tied into the vein, and heparin (3 to 5 mg/kg) administered. A No. 18-T (B-D) needle was introduced into the artery. A length of plastic tubing, attached to the hub of the arterial needle, served to conduct blood, forced by arterial pressure, several feet above the level of the operating table to the top of the resin system. The latter consisted of a 125 ml separatory funnel attached to a glass column (3.5 x 30.0 cm) through an air tight rubber stopper. The flow of blood into the separatory funnel took place through a plastic adapter fitted into an air tight stopper, which served to seal the mouth of the funnel. Blood then flowed through the stem of the funnel into the glass column. The column contained approximately 180 g of the cation exchange resin, Amberlite IR-120.† The filter unit of a Plexitron R-18 administration set (Baxter Laboratories) was fitted into the bottom of the column to prevent the escape of resin particles while blood flowed from the column through a length of plastic tubing to the No. 16 needle in the fermoral vein. Thus a continuous flow of blood from artery to vein through a closed circuit containing the resin was established. *Amberlite* IR-120 (commercial preparation) is obtainable from the source in the sodium form. For use in the resin column it was washed 10 to 15 times with distilled water, 6 times with pyrogen-free saline and autoclaved. It was again washed several times with sterile saline and poured into the column. Care was taken to prevent the collection of air bubbles within the resin. One liter of saline was then allowed to flow through the column. At the end of this washing the level of saline was adjusted at approximately 1 cm above the resin and 2 cm below the stem of the separatory funnel, making it possible to view the flow of blood into the column. A heating tape was wound spirally around the column, which was then brought to body temperature. During the experiment the temperature of the column was adjusted to compensate for heat loss from the plastic tubing and thus maintain constant body temperature. The column temperature necessary to prevent loss of body heat depended largely on room temperature and in these experiments varied from 37 to 41°C. At ordinary room temperature, failure to heat the column resulted in rapid loss of body temperature and the onset of shivering.

Results. The estimated rate of flow through the resin unit ranged from 30 to 60 ml per minute. At these rates approximately 90%

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* Sponsored by the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are a result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

† Rohm & Haas Co., Philadelphia, Pa.

TABLE I. Plasma Electrolyte Concentrations Immediately before and after Resin Therapy in 6 Uremic Dogs.

Wt, kg	K conc., mEq/l Before	K conc., mEq/l After	Na conc., mEq/l Before	Na conc., mEq/l After	Ca conc., mEq/l Before	Ca conc., mEq/l After
15	9.5	6.0	139.8	154.8		
14	11.3	7.6	146.0	156.0	4.5	3.0
25	10.8	8.0	139.2	145.8	8.0	7.5
22	8.3	5.0	148.2	159.2	7.4	3.5
20	9.1	4.7	153.8	163.8	6.4	3.9
17	9.3	7.0	137.2	141.5	4.9	3.5
8	8.0	6.1	141.0	149.4	6.0	3.8
16	10.1	4.8	146.2	162.8	6.3	4.9

of the potassium and calcium in the plasma was exchanged for an equivalent amount of sodium in one passage. Changes in plasma electrolyte concentrations which occurred as a result of 4 to 6 hours of blood circulation through the resin may be seen in Table I. In every instance there was an appreciable decrease in the plasma potassium concentration and an increase in the plasma sodium level. Decreases in plasma calcium concentration ranged from 0.5 to 3.9 mEq/l, but did not parallel decreases in the potassium levels (1.9 to 5.3 mEq/l) because of the administration of varying quantities (0 to 11.0 mEq/hr) of calcium ion during the procedure. Increases in plasma sodium concentration varied from 4.3 to 16.6 mEq/l, depending on total body potassium removed as well as the amount of calcium administered. In several instances plasma magnesium concentrations were also measured and found to decrease from 2.0 to 3.1 mEq/l.

In 4 cases platelet counts were done and there was an observed drop in the number of platelets in each case. Platelet counts never dropped below 70000 per cu mm and in none of the dogs was there any bleeding tendency after the procedure. Hematocrits and blood urea nitrogen determinations did not change as a result of treatment.

No untoward reactions were noted when the resin was prepared as described above. Gross inspection of the plasma following treatment showed no apparent hemolysis.

Discussion. The results show that elevated plasma potassium concentrations in uremic dogs may be significantly reduced by circulating their blood through a cation exchange resin. The major problem encountered with this method was the exchange of calcium and magnesium for sodium which occurred at the same time that potassium was being withdrawn. It was found that a single intramuscular injection of 25 mg per kg of magnesium sulfate was sufficient to prevent reductions in plasma magnesium levels. The control of calcium levels in the extracellular fluid is a more difficult problem. Calcium solution must be infused at a rate fast enough to prevent tetany. If it is infused too rapidly, however, an excessive amount is removed by the resin and an equally increased amount of sodium is added to the extracellular fluid. In these experiments 0 to 11.0 mEq were infused per hour, but this never was adequate to maintain the initial level. When the initial plasma sodium concentration is unduly low this may provide a safe method for raising it. When increase in the sodium concentration is not desirable, calcium replacement must be kept at a minimum.

In vitro experiments were carried out with resin which was washed with potassium-free mammalian Ringer's solution until it no longer removed calcium. The exchange capacity for potassium of 180 g of treated resin was then estimated by further washing with Ringer's solution to which potassium was added. Repeated determinations of the potassium showed that its removal from the first 2 liters of solution was virtually complete. The percentage of potassium removed from solution then progressively diminished, the total exchange capacity of 180 g of treated resin being only 20 mEq. If blood were circulated through treated resin, therefore, the capacity of the column to remove potassium would be exhausted so rapidly that frequent changing of columns would be necessary and the procedure would become impractical.

Summary. A method for removing potassium from uremic dogs by continuous circulation of blood through a cation exchange resin has been described. This method has been shown to reduce the extracellular potassium concentration by as much as 50% within 4 to 6 hours. However, it is necessary to replace calcium to prevent tetany when this method is used.

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Received October 30, 1953. P.S.E.B.M., 1953, v84.

Local Action of Adrenocortical Preparations on Secondary Sexual Organs.* (20695)

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The known local effects of adrenocortical steroids include histological alteration of the skin(1), suppression of hair growth(2,3) and of wound healing(4), and alleviation of inflammation resulting from a number of causes (5-7). Local effects on the reproductive organs have not been demonstrated. Three experiments will be described in this report which are concerned with (a) the local action of adrenocortical extract (ACE) on the vaginal mucosa, (b) the effect of intravaginal application of cortisone on stimulation of the vaginal mucosa elicited by subcutaneously injected estrone, and (c) the effect of direct application of cortisone on stimulation of the chick comb elicited by subcutaneously injected testosterone propionate (TP).

Procedure. For study of the local action of ACE† on the vaginal mucosa, 0.1 ml of hog adrenal extract (equivalent to 100 μg of cortisone as determined by the liver glycogen test) or of the solvent, 25% ethanol, was introduced daily into the vagina of adult Long-Evans rats which had been ovariectomized 8-13 days previously (Table I). Daily vaginal smears were made and the vaginae prepared for histologic study at the end of the experiment. For the study of the antagonism of cortisone to estrone, 16 young adult Long-Evans rats, which had been ovariectomized 90 days previously, were primed with 14 μg of estrone, half being given subcutaneously on each of 2 successive days. Only rats which showed cornification within 48 hours were used in the subsequent study. Vaginal smears were made 10 days after the last priming injection to ensure that the estrogenic effect had subsided. On the next day the rats were divided into 4 groups and treated as follows: (a) estrone subcutaneously, cortisone intravaginally; (b) estrone subcutaneously, saline vehicle intravaginally; (c) peanut oil subcutaneously, cortisone intravaginally; and (d) peanut oil subcutaneously, saline vehicle intravaginally. One-tenth ml of estrone (3 μg) or of peanut oil was injected subcutaneously on the 12th and 13th day after priming, 6 μg of estrone being just sufficient to produce full cornification in the primed rats as determined by previous experiments. Intravaginal injections of cortisone or saline vehicle were made with a short, blunted hypodermic needle to which a shield was attached. The cortisone was prepared by suspending 80 mg in a vehicle con-

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* This investigation supported (in part) by research grants from The National Institutes of Health, the University of Michigan Memorial-Phoenix Project and The Upjohn Co.

† We wish to thank Drs. Eldon Nielson and W. J. Haines of The Upjohn Co. for ACE; Dr. George H. Berryman of Abbott Laboratories for the estrone; Dr. Elmer Alpert, Merck & Co. Inc., for cortisone; and Dr. Cornelius Sullivan, Ciba Pharmaceutical Products, Inc., for the testosterone propionate (Perandren).

TABLE I. Effect of Daily Intravaginal Application of Adrenocortical Extract and Ethanol.

Treatment	No. of rats	% cornified	Mean No. days before cornified	Total days, Rx	Mean organ wt (mg)		
					Uterus	Thymus	Adrenal
ACE	22	100	4	8	94 ± 4.1*†	266 ± 21.9	56 ± 5
25% ethanol	20	0	—	8	71 ± 8.1‡	327 ± 73	55 ± 4.1

* 5 rats. † Stand. dev. ‡ 4 rats.

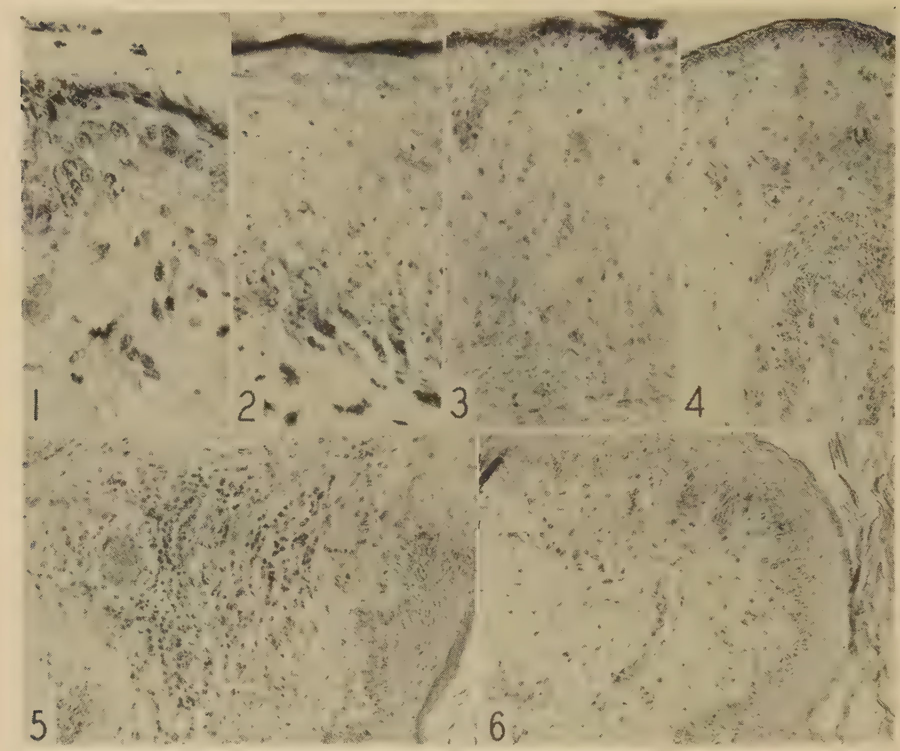
sisting of 2 drops of Tween 80 and 16 cc of 0.9% NaCl solution. One-tenth ml of cortisone or of the saline vehicle was administered 3 times daily from the 11th through the 14th day after priming. Vaginal smears were taken in the morning and evening of each day prior to the intravaginal treatment. All animals were sacrificed on the 15th day and the vaginae prepared for histologic study. For study of the effect of direct application of cortisone on stimulation of the chick's comb by TP, 2-3 day-old male Barred Rock chicks were treated as follows, the doses of TP and peanut oil being given subcutaneously once daily and those of cortisone and ethanol being applied directly to the comb thrice daily: (a) 10 chicks received 150 μg TP in sesame oil (.075 ml) and 40 μg cortisone (.02 ml) in 50% ethanol, (b) 10 received 150 μg TP and .02 ml 50% ethanol and (c) 9 received .075 ml sesame oil and .02 ml 50% ethanol. After 8 days the chicks were killed and the combs removed and weighed according to the method of Frank *et al.*(8).

Observations. (a) *ACE and vaginal mucosa.* After 8 days of treatment, the vaginal mucosa of all rats receiving ethanol intravaginally remained in the condition characteristic of ovariectomy as shown by diestrous vaginal smears and the histology of the vaginal mucosa (Fig. 1). The mucosae of all rats receiving ACE were cornified after an average of 4 days of treatment (Table I) and the epithelium was hyperplastic (Fig. 2). Weight of the adrenal glands was unchanged by treatment with ACE, but that of the thymus was reduced and of the uterus, increased.

(b) *Subcutaneous estrone and intravaginal cortisone.* The control group which received peanut oil subcutaneously and saline vehicle intravaginally showed no stimulation of the vaginal epithelium as determined by observation of vaginal smears or of the histologic sections. When peanut oil was given subcutaneously and cortisone intravaginally, no change from the ovariectomized condition was observed in the smears or histology of the vaginal epithelium. However, the concentration of cells in the lamina propria, especially of fibroblasts, was reduced and the connective tissue fibers were somewhat condensed (Fig. 4) as compared with rats which received saline intravaginally (Fig. 3). Intravaginal administration of saline vehicle did not modify the action of subcutaneously injected estrone, all rats showing full cornification 48 hours after the last injection of estrone (Fig. 5). Many mononuclear cells had infiltrated into the perivascular connective tissue of the lamina propria. Intravaginal application of cortisone failed to retard the cornification or hyperplasia of the vaginal epithelium induced by subcutaneously injected estrone but suppressed strikingly the perivascular infiltration of mononuclear cells into the lamina propria (Fig. 6).

(c) *Testosterone, cortisone, and chick's comb.* Table II demonstrates that the amount of TP injected caused a significant increase in weight of the comb and that treatment of the comb with cortisone suppressed this stimulation to a slight but statistically insignificant degree.

Discussion. The demonstration of estrogenic activity in ACE supports previous work in which estrone was isolated from the adrenal gland(9), and subcutaneous injection of ACE stimulated uterine growth(10,11) and inhibited secretion of gonadotrophin by the anterior hypophysis(12). Since most of the adrenocortical activity of our extract was due to the presence of the most potent growth inhibitor of the known adrenocortical compounds (17-hydroxycorticosterone(13)), it is surprising that the capacity of estrogen to



All figures illustrate the vaginal mucosa.

FIG. 1. One-tenth ml ethanol intravag. daily for 8 days.
FIG. 2. " " ACE for 4 days.
FIG. 3. Peanut oil subcut. and saline intravag.
FIG. 4. " " " " cortisone " .
FIG. 5. Estrone " " saline " .
FIG. 6. " " " cortisone " .

stimulate hyperplasia of the vaginal epithelium was not over-ridden. The presence of significant estrogenic activity in ACE must be taken into account in comparing the action of these preparations with that of any pure adrenocortical compound.

The failure of cortisone to stimulate the vaginal mucosa by local action supports the finding that no estrogenic activity is observed when this hormone is administered subcutaneously(14). The failure of cortisone to inhibit stimulation of the vaginal epithelium by subcutaneously injected estrogen and of the adrenocortical compounds in ACE to over-ride estrogenic activity present in the extract is due undoubtedly to the extreme sensitivity of the vaginal epithelium to the growth-stimulating action of estrogen. On the contrary others(14,15) have shown that estrogenic stimulation of the uterus may be antagonized by cortisone when it is administered *systemically*. The inhibition by cortisone of cellular infiltration of mononuclear cells into connective tissue of the lamina propria illustrates an action of the hormone which has been demonstrated under many circumstances(6).

The failure of local cortisone to inhibit growth of the chick's comb is in agreement with the results of Schiller *et al.*(16). Their experiment was repeated because they applied cortisone when dissolved in corn oil. Previous studies in this laboratory(17) showed that when adrenocortical compounds are dissolved in oils their growth-inhibiting capacity as determined by inhibition in rate of hair growth and of wound healing is relatively weak.

Summary. Under conditions of these ex-

TABLE II. The Effect of Subcutaneous TP and Local Cortisone on Weight of Chick's Comb.

Group	Mean body wt (g)	Mean comb wt (mg)	Comb wt (mg) / Body wt (g)
TP + cortisone	71 ± 8.1*	62.6 ± 12.9 $P<.3$†	.86 ± .15 $P<.5$
TP + ethanol	78 ± 8.5	73.6 ± 25.6	.94 ± .30
Ses. oil + ethanol	73 ± 11.4	23.3 ± 36.6	.31 ± .06

* Stand. dev. † Fisher student t test.

periments adrenocortical steroids failed to suppress the growth-stimulating action of sex hormones on secondary reproductive organs as revealed by hyperplasia and cornification of the vaginal epithelium which resulted from direct application of ACE, by failure of locally applied cortisone to suppress estrogenic stimulation of the vagina and of androgenic stimulation of the chick's comb.

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Received October 30, 1953. P.S.E.B.M., 1953, v84.

Comparison of *in vitro* Serum Protein Binding of Thyroxin and Triiodothyronine.* (20696)

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(Introduced by Ovid O. Meyer.)

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It has been observed that 3:5:3′ L triiodothyronine disappears more rapidly than L thyroxin from blood following intravenous administration(1). It is possible that this observation may be explained by a difference in serum protein binding of these two amino acids inasmuch as thyroxin, but not triiodothyronine, has been shown to associate with a specific alpha globulin *in vivo*(2).

The purpose of this paper is to report on the *in vitro* binding of L thyroxin and 3:5:3′ L triiodothyronine by serum proteins.

Methods and materials. The radioactive amino acids† employed in these experiments were of sufficiently high specific activity to permit the use of quantities that were approxi-

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* Supported in part by Wisconsin Alumni Research Foundation.

† The radiothyroxin‡ used in these studies contained on chromatographic analysis nearly 100% of its radioactivity as thyroxin. The radio triiodothyronine‡ was found to contain approximately 5% of its radioactivity as thyroxin.

‡ Obtained from Abbott Laboratories.

TABLE I. Mixtures of Labelled and Unlabelled Amino Acids Added to Serum in Serum Protein Binding Experiments.

	μg of amino acid/ml serum			
Exp.	Radioactive thyroxin	Thyroxin	Radioactive triiodo-thyronine	Triiodo-thyronine
I	.1	—	—	—
II	.1	—	—	.1
III	.1	—	—	1.0
IV	—	—	.1	—
V	—	.1	.1	—
VI	—	1.0	.1	—

mately physiological. The labelled amino acids were dissolved in sodium hydroxide. Stock solutions were then prepared to contain 1.0 μg/ml in 0.001 N NaOH. Solutions of unlabelled amino acids§ containing 1.0 and 10 μg/ml in 0.001 N NaOH were prepared. As a preliminary experiment 0.1 ml of the labelled amino acid solution was added to 1.0 ml of saline and subjected to electrophoresis as described below. In the serum experiments quantities of the radioactive and unlabelled amino acid seen in Table I were added to 1 ml of serum. In each instance the unlabelled analogue was added 15 minutes after the labelled amino acid. This amino acid serum mixture was allowed to stand for 15 minutes, following which 0.05 ml was subjected to electrophoresis as previously described(3). Whatman No. 3 MM paper was used for these studies. The electrophoresis was carried out at pH 8.6, using 0.075 M veronal buffer and at pH 7.4, using 0.075 M phosphate buffer. Upon completion of electrophoresis the paper strips were dried, sectioned into 1 cm segments and counted. Protein zones were localized by bromphenol blue staining.

Results. In the electrophoresis of the amino acids in saline solution (Fig. 1), triiodothyronine showed no movement while thyroxin moved slightly toward the anodal end of the paper strip. The electrophoresis of the amino acid-serum mixtures at pH 7.4 and 8.6 disclosed a definite and consistent localization of both amino acids to an alpha globulin (thyroxin-binding protein) just ahead of the alpha-2 globulin zone (Fig. 2, I and IV). A small fraction of radioactivity remained at the starting point of electrophoresis and in the thyroxin experiment a small fraction moved with albumin. There was much greater trailing of the triiodothyronine than of the thyroxin. It is believed that this trailing of triiodothyronine represents dissociation of the amino acid from the thyroxin-binding globulin as this protein moved along the paper. That it is not "non-specific" binding with the various proteins in this zone is indicated by the lack of correlation between the distribution of the radioactivity

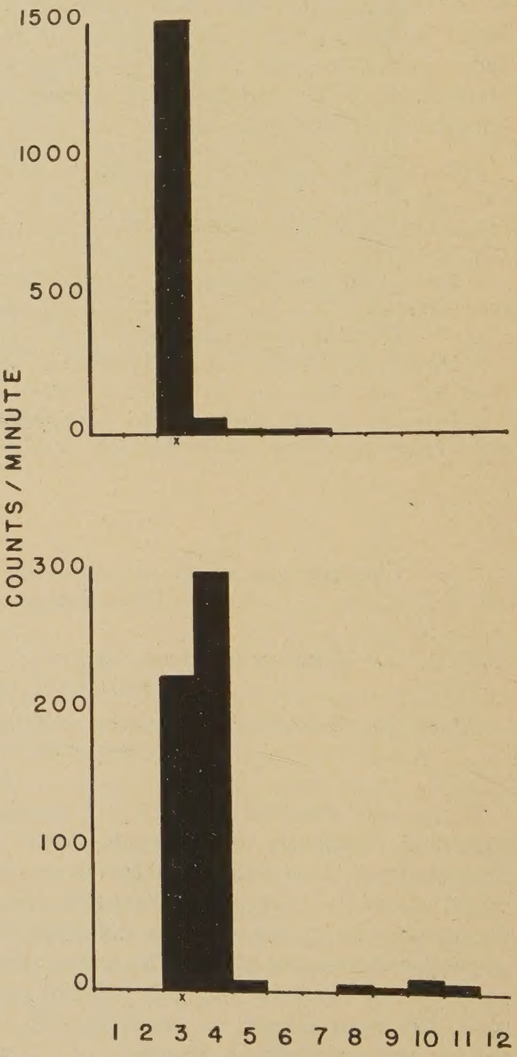

FIG. 1. Radioactivity in 2 cm segments of paper after electrophoresis in veronal buffer of saline solutions of radioactive triiodothyronine (top graph) and radioactive thyroxine (lower graph). (X) marks the starting point of electrophoresis.

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§ Thyroxin was kindly supplied by E. R. Squibb & Sons. Triiodothyronine was kindly supplied by Smith, Kline & French Laboratories.

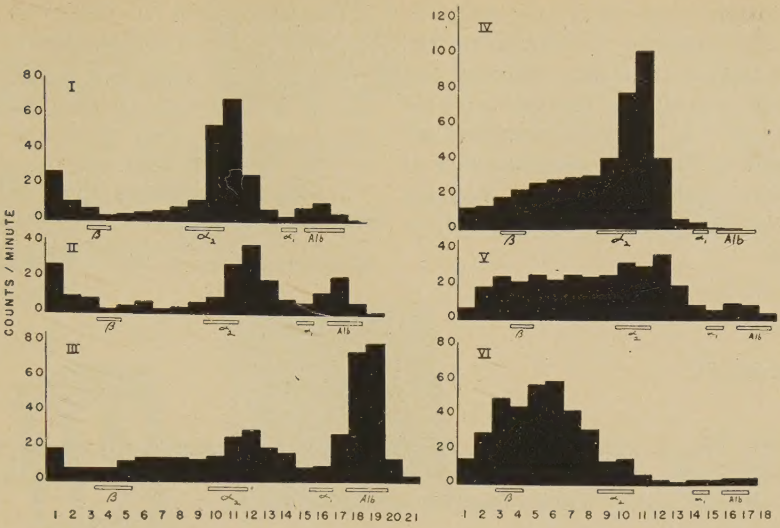

FIG. 2. Distribution of radioactivity in 1 cm segments of paper after electrophoresis in veronal buffer of the amino acid-serum mixtures described in Table I. The protein fractions are marked schematically by the small boxes. Only the paper on the anodal side of the starting point is shown since there was never any radioactivity in the gamma globulin, which moves by electro-osmosis toward the cathode.

and the concentration of the protein in this zone.

Fig. 2 (II and V) represent the effect of the addition of equal quantities of the unlabelled analogue to serum containing the radioactive thyroxin or triiodothyronine. The presence of unlabelled thyroxin greatly increases the dissociation of triiodothyronine whereas the binding of radioactive thyroxin is only slightly affected by the addition of triiodothyronine.

Fig. 2 (III and VI) represent the effect of the addition of excess quantities of the unlabelled analogue to serum containing the radioactive thyroxin or radioactive triiodothyronine. The addition of unlabelled thyroxin in quantities 10 times as great as radioactive triiodothyronine prevents entirely the association of radioactive triiodothyronine with the thyroxin-binding globulin while the presence of 10 times the quantity of unlabelled triodothyronine fails to displace radioactive thyroxin completely from this protein.

There is a notable difference in the behavior of the displaced labelled amino acids. While displaced radioactive triiodothyronine does not associate with any specific protein, displaced radioactive thyroxin becomes associated with albumin.

Discussion. The demonstration of the *in vitro* association of thyroxine with the thyroxin-binding globulin is consistent with previous observations that thyroxin is associated with this protein *in vivo*(2). It is not unexpected that triiodothyronine also associates with this protein *in vitro* in view of the similarity of their molecular structures.

It appears that the thyroxin-binding protein has a greater affinity for thyroxin than for triiodothyronine, as thyroxin will displace triiodothyronine from this linkage. It is possible, in view of the known presence of thyroxin in serum in quantities greater than triiodothyronine, that thyroxin blocks the binding of triiodothyronine with this protein or replaces it once bound. This may account for a greater diffusibility of triiodothyronine and hence its more rapid disappearance from serum.

Summary. 1. *In vitro* experiments on the serum protein binding of thyroxin and triiodothyronine were performed using paper electrophoresis to separate the protein fractions and I^{131} labelled triiodothyronine and thyroxin to trace the localization of these amino acids. 2. Although both thyroxin and triiodothyronine were shown to be bound by alpha globulin, thyroxin was more firmly bound and not easily displaced by triiodothyronine whereas triiodothyronine was less firmly

bound and readily displaced by thyroxin. 3. Displaced triiodothyronine was found to trail the thyroxin-binding globulin, whereas displaced thyroxin was seen to associate with the albumin fraction. 4. These observations are possibly related to the rapid disappearance of triiodothyronine after intravenous injection and to the relatively low levels of triiodothyronine in blood.

The authors wish to acknowledge the invaluable assistance of Miss Sue Ames, Medical Technician.

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Received October 26, 1953. P.S.E.B.M., 1953, v84.

Erratum

Vol. 84, No. 1, Article 20539, Transfusion of Separated Leucocytes into Irradiated Dogs. . ., by Brecher *et al.*, page 56, left column, 12th line from bottom should read, "agranulocytic tonsillar lesion . . ."